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(54) Title: CTL EPITOPES FROM EBV

(57) Abstract

The present invention provides cytotoxic Epstein–Barr virus (EBV) T–cell epitopes derived from EBV structural antigens. Preferred epitopes include YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29). The present invention also provides methods of treating or preventing EBV infection in subjects which involve administration of EBV cytotoxic T–cell epitopes.

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CTL epitopes from EBV

FIELD OF THE INVENTION

The present invention relates to methods of treating or preventing EBV infections. The present invention also relates to cytotoxic T-cell (CTL) epitopes within Epstein-Barr virus (EBV) structural and latent antigens, and to subunit vaccines and nucleic acid vaccines which include these epitopes.

BACKGROUND OF THE INVENTION

It is now well established that long-term protection from persistent viral infection requires the development of virus-specific memory T cells which recognize viral antigens in association with either class I or class II MHC molecules. Since immunization with whole viral proteins is unable to elicit an efficient CTL response, interest has been directed towards designing vaccines based on defined epitope sequences. This is particularly the case with oncogenic viruses, since individual viral genes introduced in recombinant vectors have the potential to initiate tumorgenic processes. Two broad approaches are currently being considered to design an effective vaccine for controlling Epstein-Barr virus (EBV) associated diseases (for review see ref. (7)). These include directing immune responses to either EBV structural antigens or latent antigens.

In the last few years, most of the vaccine development efforts have concentrated on the use of a subunit preparation of gp350 (recombinant and affinity purified) and have been directed towards blocking virus attachment to the target cell in the oropharynx (31). The general approach has been to immunize cotton-top marmosets with gp350 and determine their ability to restrict the outgrowth of EBV-positive lymphomas in these animals. Indeed, highly purified gp350, when administered subcutaneously in conjunction with adjuvants (muramyl dipeptide or ISCOMS), induced high levels of serum neutralizing antibodies and inhibited tumor formation in cotton-top tamarins (32). A number of recombinant vectors including, vaccinia-gp350 and adenovirus 5-gp350 have also been successfully used in these animals to block tumor outgrowth (33). The precise mechanism by which gp350 affords protection from lymphomas in cotton-top tamarins remains unclear. The fact that development of neutralizing antibody titres in vaccinated animals does not always correlate with protection indicates that gp350-specific T cell-mediated immune responses may also have an effector role (34). Furthermore, Yao and colleagues (35) showed that very low levels of

neutralizing anti-gp350 antibodies are present in the saliva of healthy EBV-immune donors, which suggests that such antibodies are unlikely to be the basis of long-term immunity in healthy seropositive individuals. It has been postulated that gp350 specific T cell -mediated immune responses may have an effector role in protection. There has been no identification to date, however, of CTL epitopes within the EBV structural antigens.

Post-transplant lymphoproliferative disease (PTLD) that arises in organ transplant patients is an increasingly important clinical problem. Histological analysis of PTLD shows a quite complex clonal diversity ranging from polymorphic B lymphocyte hyperplasia to malignant monoclonal lymphoma. This range of pathology encompasses the collective term PTLD while the lymphomas are frequently referred to as immunoblastic lymphomas(IL). This condition is clearly associated with the proliferation of Epstein-Barr virus (EBV) infected B cells which are carried for life in all previously infected individuals (about 80% of adults and 20% of children 7 years) (45, 46, 47, 49). These EBV-infected B cells are normally restricted in their growth in vitro and in vivo by virus-specific cytotoxic T cells (CTLs) which recognise epitopes included within the EBV latent proteins (see below) (48). Immunosuppression inhibits these specific CTL and results in an expansion of the pool of EBV-infected B cells and the emergence of the clinical problems associated with PTLD. It is known that the individuals at greatest risk of PTLD are EBV seronegative recipients who receive a transplant from a seropositive donor (Crawford and Thomas, 1993). Immunisation of EBV seronegative graft recipients prior to engraftment will greatly reduce the risk of PTLD.

The role of the immune system in the rejection of virus-associated cancers has also been the subject of intense study recently. The hypothesis under investigation is that many neoplasms express viral antigens that should potentially enable them to be recognized and destroyed by the immune system, including both T helper cells and cytotoxic T lymphocytes (CTL). There is now compelling evidence that most of the Epstein-Barr virus (EBV)-associated malignancies escape this potent virus-specific CTL response by restricting viral gene expression (7,20,21). For malignancies such as nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD), EBV nuclear antigen 1 (EBNA1) and latent membrane protein 1 (LMP1) are the only antigens consistently expressed and are therefore the potential target

antigens for any future vaccine designed to control these tumors (3,28). Since it is well established that immunization with whole viral proteins does not elicit an efficient CTL response, interest has been directed towards developing peptide vaccines based on defined epitope sequences.

SUMMARY OF THE INVENTION

Results obtained by the present inventors indicate that CTL epitopes within EBV structural and latent proteins may be effective in providing antiviral immunity against EBV infection. In particular, the present inventors have analysed the latent antigen LMP1 sequence, using peptide stablization assays, and found that this antigen includes potential CTL epitopes. Following *in vitro* activation with these peptides, both polyclonal and clonal CTLs from HLA A2-positive donors showed strong reactivity against target cells expressing the LMP1 antigen. Moreover, lymphoblastoid cell lines (LCL), expressing different HLA A2 supertypes were efficiently recognized by these CTLs, a result that has important implications for the design of an anti-viral vaccine aimed at protecting different ethnic populations.

The present inventors have also found that CTLs from acute infectious mononucleosis (IM) patients display strong reactivity against the EBV structural antigens gp85 and gp350. In addition, specific CTL epitopes within EBV structural antigens gp85 and gp350 have been identified for the first time. Importantly, prior immunisation of HLA A2/K^b transgenic mice with these gp350 and gp85 CTL epitopes induced a strong epitope-specific CTL response and afforded protection against gp85- or gp350-expressing vaccinia virus challenge. These results provide evidence, for the first time, of the existence of CTL epitopes in EBV structural proteins and show that they may be used for establishing strong anti-viral immunity against EBV infection.

Accordingly, in a first aspect the present invention provides a cytotoxic Epstein-Barr virus (EBV) T-cell epitope, the epitope being derived from an EBV structural antigen.

In a preferred embodiment of the first aspect of the present invention, the EBV structural antigen is gp85 or gp350.

In a second aspect the present invention provides a cytotoxic Epstein-Barr virus T-cell epitope, the epitope being selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32),

YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

In a third aspect the present invention provides a subunit vaccine including a cytotoxic Epstein-Barr virus (EBV) T-cell epitope according to the first aspect of the present invention.

In a preferred embodiment, the subunit vaccine includes at least one T-cell epitope selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

In a preferred aspect of the present invention the epitope is selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YLQQNWWTL (SEQ ID NO:6), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), and VLQWASLAV (SEQ ID NO:27).

In a further preferred embodiment, the subunit vaccine includes one or more additional cytotoxic EBV T-cell epitopes. The additional cytotoxic EBV T-cell epitope(s) may be selected from those described in WO 97/45444, the entire contents of which are incorporated herein by reference.

In a further preferred form of the present invention the vaccine includes a water-in-oil formulation. It is further preferred that the vaccine includes at least one antigen to which the individual will mount an anamnestic response in addition to the at least one cytotoxic T-cell epitope.

The at least one antigen is preferably selected from the group consisting of tetanus toxoid, diphtheria toxoid, Bordetella pertussis antigens,

poliovirus antigens, purified protein derivative (PPD), gp350 protein (Thorley-Lawson, D.A. and Poodry, C.A. (1982). Identification and isolation of the main component (gp350-gp220) of Epstein-Barr virus responsible for generating neutralizing antibodies in vivo. J. Virol. 43, 730-736), helper epitopes and combinations thereof and is preferably tetanus toxoid.

It is preferred that the water-in-oil formulation is Montanide ISA 720. Additional information regarding this formulation can be found in WO 95/24926, the disclosure of which is incorporated herein by cross reference.

The subunit vaccine may also be formulated using ISCOMs. Further information regarding ISCOMs can be found in Australian Patent Nos. 558258, 590904, 632067, 589915, the disclosures of which are included herein by cross reference.

In a fourth aspect the present invention provides an isolated nucleic acid sequence encoding a cytotoxic Epstein-Barr virus (EBV) T-cell epitope according to the first aspect of the present invention.

In a preferred embodiment, the isolated nucleic acid sequence encodes at least one of the cytotoxic T-cell epitopes selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

As will be appreciated by those skilled in the field the nucleic acid sequence can be delivered as naked nucleic acid or using a suitable viral or bacterial vectors. Suitable bacterial vectors include the bacteria *Salmonella spp.*. Suitable viral vectors include, for example, retroviral vectors, adenoviral vectors and vaccinia vectors. An example of a suitable vaccinia vector is a modified Vaccinia Ankara vector.

Vectors suitable for delivery of nucleic acid sequences have previously been described. For example, alphavirus vectors have become

widely used in basic research to study thestructure and function of proteins and for protein production purposes. Development of a variety of vectors has made it possible to deliver foreignsequences as naked RNA or DNA, or as suicide virus particles produced using helper vector strategies. Preliminary reports also suggest that these vectors may be useful for in vivo applications where transient, high-level protein expression is desired, such as recombinant vaccines. The initial studies have already shown that alphavirus vaccines can induce strong humoral and cellular immune responses with good immunological memory and protective effects. See, for example, Tubulekas I., Berglund P., Fleeton M., and Liljestrom P. (1997) Alphavirus expression vectors and their use as recombinant vaccines: a minireview, *Gene* 190(1):191-195.

Recombinant pox viruses have been generated for vaccination against heterologous pathogens. Amongst these, the following are notable examples. (i) The engineering of the Copenhagen strain of vaccinia virus to express the rabies virus glycoprotein. When applied in baits, this recombinant has been shown to vaccinate the red fox in Europe and raccoons in the United States, stemming the spread of rabies virus infection in the wild. (ii) A fowlpox-based recombinant expressing the Newcastle disease virus fusion and hemagglutinin glycoproteins has been shown to protect commercial broiler chickens for their lifetime when the vaccine was administered at 1 day of age, even in the presence of maternal immunity against either the Newcastle disease virus or the pox vector. (iii) Recombinants of canarypox virus, which is restricted for replication to avian species, have provided protection against rabies virus challenge in cats and dogs, against canine distemper virus, feline leukemia virus, and equine influenza virus disease. In humans, canarypox virus-based recombinants expressing antigens from rabies virus, Japanese encephalitis virus, and HIV have been shown to be safe and immunogenic. (iv) A highly attenuated vaccinia derivative, NYVAC, has been engineered to express antigens from both animal and human pathogens. Safety and immunogenicity of NYVAC-based recombinants expressing the rabies virus glycoprotein, a polyprotein from Japanese encephalitis virus, and seven antigens from Plasmodium falciparum have been demonstrated to be safe and immunogenic in early human vaccine studies. See, for example, Paoletti E. (1996) Applications of

pox virus vectors to vaccination: an update, *Proc Natl Acad Sci U S A*, 93(21):11349-11353.

Progress towards effective vaccines to control internal parasites, especially those affecting mucosal compartments, has been inhibited by the combined problems of the antigenic complexity of parasites and the lack of understanding of the host response. However, the accumulation of information regarding regulation of mucosal immunity has enabled a reappraisal of vaccination options to provide appropriate mucosal effector responses. The pivotal role of T cell influences, and in particular the contribution of cytokine signals, has been clearly established from in vitro studies, but data emerging from our laboratories provide evidence for these effects in vivo. We have demonstrated the role of T cells in determining the outcome of an intestinal response and propose a role for local Th2 cytokine production in this regard. To support this proposition, the distribution of cytokine mRNA has been determined by in situ hybridisation techniques in normal and parasitised animals. Further, we have shown that in the absence of Th2 cytokines (using gene knockout animals) mucosal responses are grossly deficient; we have also shown that this defect can be overcome by vector-directed gene therapy. These studies have indicated that new mucosal immunisation opportunities exist by combining traditional immunisation approaches with strategies to upregulate local cytokine production. However, the success of these new strategies will depend on selection of highly immunogenic subunit antigens, coupled with techniques for cytokine manipulation and delivery with appropriate adjuvant/vehicle formulations. This paper reviews delivery technologies available to chaperone labile antigenic and genetic material to appropriate sites for mucosal stimulation after systemic or oral administration. See, for example, Sutter G. et al (1994) A recombinant vector derived from the host range-restricted and highly attentuated MVA strain of vaccinia virus stimulates protective immunity in mice to influenza virus. *Vaccine* 12:1032-1040; and Husband A.J., Bao S., McClure S.J., Emery D.L., Ramsay A.J. (1996) Antigen delivery strategies for mucosal vaccines, *Int J Parasitol* 8-9:825-834.

Attenuated *Salmonella typhi* vaccine strain CVD 908, which harbors deletion mutations in aroC and aroD, has been shown to be well-tolerated and highly immunogenic, eliciting impressive serum antibody, mucosal IgA and cell-mediated immune responses. A further derivative prepared by

introducing a deletion in htrA (which encodes a heat-shock protein that also has activity as a serine protease in CVD 908 resulted in CVD 908-htrA. In phase 1 clinical trials, CVD 908-htrA appears very attractive as a live oral vaccine candidate. Both CVD 908 and CVD 908-htrA are useful as live vector vaccines to deliver foreign antigens to the immune system. Conditions that enhance the expression and immunogenicity of foreign antigens carried by CVD 908 and CVD 908-htrA are being investigated. For a review of *Salmonella* vectors, see Levine M.M., Galen J., Barry E., Noriega F., Chatfield S., Sztein M., Dougan G. And Tacket C (1996) Attenuated *Salmonella* as live oral vaccines against typhoid fever and as live vectors, *J Biotechnol* 44(1-3): 193-196.

The isolated nucleic acid sequences may be in the form of nucleic acid vaccines. Further information regarding nucleic acid vaccines can be found in WO 96/03144 and in Suhrbier A (1997), Multi-epitope DNA vaccines, *Immunol Cell Biol* 75(4):402-408 the disclosures of which are incorporated herein by cross reference.

In a fifth aspect the present invention provides an isolated polypeptide, the polypeptide including at least one epitope according to the first or second aspects of the present invention.

The vaccines of the present invention may be used prophylactically or therapeutically.

The CTL epitopes of the present invention may be synthesised using techniques well known to those skilled in this field. For example, the CTL epitopes may be synthesised using solution synthesis or solid phase synthesis as described, for example, in Chapter 9 entitled "Peptide Synthesis" by Atherton and Sheppard which is included in a publication entitled "Synthetic Vaccines" edited by Nicholson and published by Blackwell Scientific Publications. Preferably a solid phase support is utilised which may be polystyrene gel beads wherein the polystyrene may be cross-linked with a small proportion of divinylbenzene (e.g. 1%) which is further swollen by lipophilic solvents such as dichloromethane or more polar solvents such as dimethylformamide (DMF). The polystyrene may be functionalised with chloromethyl or anionomethyl groups. Alternatively, cross-linked and functionalised polydimethyl-acrylamide gel is used which may be highly solvated and swollen by DMF and other dipolar aprolic solvents. Other supports can be utilised based on polyethylene glycol which is usually

grafted or otherwise attached to the surface of inert polystyrene beads. In a preferred form, use may be made of commercial solid supports or resins which are selected from PAL-PEG, PAK-PEG, KA, KR or TGR.

In solid state synthesis, use is made of reversible blocking groups which have the dual function of masking unwanted reactivity in the a-amino, carboxy or side chain functional groups and of destroying the dipolar character of amino acids and peptides which render them inactive. Such functional groups can be selected from t-butyl esters of the structure RCO-$OCMe_3$-CO-NHR which are known as t-butoxy carboxyl or ROC derivatives. Use may also be made of the corresponding benzyl esters having the structure RCO-OCH_2-C_6H_5 and urethanes having the structure $C_6H_5CH_2O$ CO-NHR which are known as the benzyloxycarbonyl or Z-derivatives. Use may also be made of derivatives of fluorenyl methanol and especially the fluorenyl-methoxy carbonyl or Fmoc group. Each of these types of protecting group is capable of independent cleavage in the presence of one other so that frequent use is made, for example, of BOC-benzyl and Fmoc-tertiary butyl protection strategies.

Reference also should be made to a condensing agent to link the amino and carboxy groups of protected amino acids or peptides. This may be done by activating the carboxy group so that it reacts spontaneously with a free primary or secondary amine. Activated esters such as those derived from p-nitrophenol and pentafluorophenyl may be used for this purpose. Their reactivity may be increased by addition of catalysts such as 1-hydroxybenzotriazole. Esters of triazine DHBT (as discussed on page 215-216 of the abovementioned Nicholson reference) also may be used. Other acylating species are formed in situ by treatinent of the carboxylic acid (i.e. the Nα-protected amino acid or peptide) with a condensing reagent and are reacted immediately with the amino component (the carboxy or C-protected amino acid or peptide). Dicyclohexylcarbodiimide, the BOP reagent (referred to on page 216 of the Nicholson reference), O'Benzotriazole-N, N, N'N'-tetra methyl-uronium hexaflurophosphate (HBTU) and its analogous tetrafluroborate are frequently used condensing agents.

The attachment of the first amino acid to the solid phase support may be carried out using BOC-amino acids in any suitable manner. In one method BOC amino acids are attached to chloromethyl resin by warming the triethyl ammonium salts with the resin. Fmoc-amino acids may be coupled

to the p-alkoxybenzyl alcohol resin in similar manner. Alternatively, use may be made of various linkage agents or "handles" to join the first amino acid to the resin. In this regard, p-hydroxymethyl phenylactic acid linked to aminomethyl polystyrene may be used for this purpose.

As will be readily appreciated by those skilled in the art the LMP1, gp85 and gp350 epitopes and vaccines of the present invention can be used to treat and to protect against EBV. Further, given the possible greater involvement of EBV infection in immunocompromised individuals, the present invention may have particular application in the treatment and protection of individuals having decreased immune function, eg transplant patients. Importantly, the present inventors have found that EBV transformed lymphoblastoid cell lines expressing different HLA A2 supertypes are efficiently recognised by LMP1-specific CTL clones. This highlights the potential for the design of an antiviral vaccine aimed at treating and protecting different ethnic populations.

Accordingly, in a sixth aspect the present invention provides a method of preparing a composition for use in inducing CTLs in a subject, the method including admixing at least one epitope according to the first or second aspects of the present invention with at least one pharmaceutical acceptable carrier, diluent or excipient.

In a seventh aspect the present invention provides a method of reducing the risk of EBV infection in a subject which method includes administering to the subject an effective amount of:-

(1) at least one CTL epitope according to the first or second aspects of the present invention;

(2) a subunit vaccine according to the third aspect of the present invention;

(3) a nucleic acid sequence according to the fourth aspect of the present invention;

(4) a vector according to the fourth aspect of the present invention; or

(5) a polypeptide according to the fifth aspect of the present invention.

In an eighth aspect the present invention provides a method of treating or preventing nasopharyngeal carcinoma or Hodgkin's disease in a subject which method includes administering to the subject an effective

amount of at least one CTL epitope derived from an EBV structural or latent antigen.

By "effective amount" we mean a quantity of the epitope which is sufficient to induce or amplify a CTL response against an EBV antigen.

In a preferred embodiment of the eighth aspect of the present invention, the EBV structural antigen is gp85 or gp350 and the latent antigen is LMP1 or LMP2. In a further preferred embodiment the CTL epitope is an epitope as defined in the second aspect of the present invention.

The present inventors have also made the surprising finding that NPC cells which are recognised by CTL clones are subject to CTL lysis. This finding has important implications for the design of vaccines to control NPC tumours *in vivo*.

In an ninth aspect the present invention provides a method of treating or preventing growth of NPC or HD cells in a subject in need thereof which method includes administering to the subject at least one CTL epitope derived from an EBV structural or latent antigen.

In a preferred embodiment of the ninth aspect of the present invention, the EBV CTL epitopes are derived from the gp85 or gp350 antigens. In a further preferred embodiment, the EBV CTL epitopes are derived from the LMP1 or LMP2 antigens. Preferably, the CTL epitopes are derived from the LMP1 antigen.

In a tenth aspect the present invention provides a method of treating or preventing the growth of NPC or HD cells in a first subject which method includes transferring to the subject EBV-specific CTLs which recognise NPC or HD cells.

In a preferred embodiment the EBV-specific CTLs are obtained from NPC patients by *in vitro* stimulation of CTLs by exposure to EBV CTL epitopes. Alternatively, the EBV-specific CTLs may be obtained from a second subject, wherein the second subject is infected with EBV but does not have NPC.

In a further preferred embodiment of the tenth aspect of the present invention, the EBV-specific CTLs are LMP1 and/or LMP2-specific CTLs.

In an eleventh aspect the present invention provides a method of reducing the risk of infectious mononucleosis or post transplantation lymphoproliferative disease in a subject which method includes administering to the subject an effective amount of:-

(1) at least one CTL epitope according to the first or second aspects of the present invention;

(2) a subunit vaccine according to the third aspect of the present invention;

(3) a nucleic acid sequence according to the fourth aspect of the present invention;

(4) a vector according to the fourth aspect of the present invention; or

(5) a polypeptide according to the fifth aspect of the present invention.

The terms "comprise", "comprises" and "comprising" as used throughout the specification are intended to refer to the inclusion of a stated component or feature or group of components or features with or without the inclusion of a further component or feature or group of components or features.

In order that the nature of the present invention may be more clearly understood forms thereof will now be described with reference to the following examples and figures.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1: MHC stablization analysis on T2 cells using potential HLA A2 binding peptides within LMP1. T2 cells were initially incubated with 200µl of each of the peptides (200µg/ml) for 14-16h at 26°C followed by incubation at 37°C for 2-3h. HLA A2 expression on these cells was analysed by FACS using the BB7.2 antibody. The dotted line indicates the background mean fluorescence intensity for HLA A2 on T2 cells without any peptide. The LMP1 peptides showing significant stablization of HLA A2 molecules on T2 cells are indicated by arrows.

Figure 2: Recognition of LMP1 peptides by polyclonal CTLs from an HLA A2-positive donor SB. PBMC from donor SB were co-cultivated for seven days with irradiated T2 cells sensitized with synthetic peptides (indicated on the Y-axis). On day 10, these cells were used as polyclonal effectors in a standard ^{51}Cr-release assay against peptide-sensitized (1µg/ml) autologous PHA blasts. An effector:target ratio of 20:1 was used in the assay. Data from one representative experiment out of three is shown. Results are expressed as percent specific lysis.

Figure 3: Specific lysis by an EBV-specific CTL clone (SB7) from donor SB of autologous LCLs and autologous CD40 B cells infected with Vacc.EBNA1, 2, 3, 4, 5, 6, LMP1, LMP2A and Vacc.TK-(panel A). Target cells were infected for 12-14 h (M.O.I = 10:1) with vaccinia constructs and processed for the standard ^{51}Cr-release assay. Vacc.TK- was used as a control recombinant vaccinia. To further confirm the LMP1 specificity of the SB7 clone, LMP1-and HLA A2-positive BL cell lines (BJAB.MTLM6 or Mutu cl.59) and LMP1-negative, HLA A2-positive BL cell lines (BJAB.gpt1 and Mutu cl.216) were used as targets in a standard ^{51}Cr-release assay (panel B). An effector:target ratio of 4:1 was used in both assays. Results are expressed as percent specific lysis.

Figure 4: CTLp frequencies for the LMP1 epitopes YLQQNWWTL (SEQ ID NO:6) (A) and YLLEMLWRL (SEQ ID NO:1) (B) in HLA A2-positive donor are shown. Using limiting dilution analysis, the frequencies of CTLp for peptides YLQQNWWTL (SEQ ID NO:6) (A) and YLLEMLWRL (SEQ ID NO:1) (B) were estimated in peripheral blood lymphocytes from donor SB. PBMC from this donor was stimulated with peptides sensitized PBMC as described in the "Materials and Methods" section. Reciprocal values of responder frequencies (f^{-1}) are indicated. The shaded areas indicate 95% confidence limits.

Figure 5: CTL recognition of LCLs expressing different supertypes of HLA A2 by the SB7 CTL clone. All LCLs used in this assay were transformed with the B95.8 EBV isolate. The HLA A2 subtyping for each LCL was assigned by the 12th Histocompatability workshop. An effector:target ratio of 4:1 was used in both the assay. Results are expressed as percent specific lysis. Data from one representative experiment out of four is shown.

Figure 6: Effect of variant LMP1 peptides on the HLA A2 binding (panel A) and CTL recognition (panel B). For HLA A2 binding analysis on T2 cells were initially incubated with 200µl of each of the peptides (200µg/ml) for 14-16h at 26^{0}C followed by incubation at 37^{0}C for 2-3h. HLA A2 expression on these cells was analysed by FACS using BB7.2 antibody (panel A). The dotted line indicates the background mean fluorescence intensity for HLA A2 on T2 cells without any peptide. For CTL recognition of the variant and prototypic HLA A2-restricted LMP1 epitope, PHA blasts from donor SB were sensitized with serial dilutions of each of the peptides and then exposed to the SB7 CTL clone (panel B) at an effector:target ratio of

4:1. Results are expressed as percent specific lysis. Data from one representative experiment out of three is shown. Amino acid changes in the variant peptides are indicated by bold letters.

Figure 7: CTL recognition of NPC cells and LCLs by EBV-specific HLA A11-restricted CTL clones CM9 (a & c) and CM29 (b & d). Vacc.EBNA4, Vacc.TK infected or peptide sensitized C15NPC cells (a & b) were exposed to CTL clones at different effector to target ratios and the level of CTL lysis was compared to a type 2 LCLs (CM/Ag876 LCL). As a positive control target cells were either presensitized with IVTDFSVIK peptide (a & c), or AVFDRKSDAK peptide (b & d). The results were expressed as per cent specific lysis.

Figure 8: MHC stabilization analysis on T2 cells using potential HLA A2 binding peptides within gp85 (panel A) and gp350 (panel B). T2 cells were initially incubated with 200Tl of each of the peptides (200Tg/ml) for 14-16h at 26°C followed by incubation at 37°C for 2-3h. HLA A2 expression on these cells was analysed by FACS using the BB7.2 antibody. The dotted line indicates the background mean fluorescence intensity for HLA A2 on T2 cells without any peptide. The gp85 and gp350 peptides showing significant stabilization of HLA A2 molecules on T2 cells are indicated by arrows.

Figure 9: gp85 and gp350-specific ex vivo cytotoxic T cell activity in peripheral blood lymphocytes from IM donors. Panel A, B & C shows ex vivo CTL lysis of peptide-sensitized (1ug/ml) PHA blasts at two different effector:target (E/T) ratios. Data from IM patient SB, LP and MG are presented in panel A, B and C respectively. Panel D shows ex vivo CTL lysis by peripheral blood lymphocytes from patient LP of target cells infected with recombinant vaccinia encoding either gp85 (Vacc.gp85) or gp350 (Vacc.350) by . Vacc.TK- and Vacc.EBNA2 were used as control in the assay.

Figure 10: Recognition of gp85 and gp350 peptides by polyclonal CTLs from an HLA A2-positive IM recovered (36 months post IM) individual. PBMC were co-cultivated for seven days with irradiated T2 cells sensitized with synthetic peptides (indicated on the Y-axis). On day 18, these cells were used as polyclonal effectors in a standard 51Cr-release assay against peptide-sensitized (1Tg/ml) autologous PHA blasts. An effector:target ratio of 20:1 was used in the assay. Individual peptide stimulated CTL effectors used in this experiments are indicated in the figure. Data from one representative

experiment out of three is shown. Results are expressed as percent specific lysis.

Figure 11: Immunization of HLA A2/K^b mice with gp85 and gp350 CTL epitopes induces strong CTL response. Animals were twice immunized (at a 14 day interval) subcutaneously with individual CTL epitope with Tetanus Toxoid as a source of help. Four weeks following peptide immunization, animals were assessed for gp350- and gp85-specific CTL response. Panel A, B & C shows CTL activity in splenocytes from two mice immunized with VLQWASLAV (SEQ ID NO:27) (gp350), TLFIGSHVV (SEQ ID NO:24) (gp85) and SLVIVTTFV (SEQ ID NO:17) (gp85) respectively. CTL lysis of target cells sensitized with peptide epitopes are shown as filled symbols, while lysis of unsensitized target cells is shown as empty symbols. Panel D shows CTL activity in pooled inguinal lymphnodes from mice immunised with peptide VLQWASLAV (SEQ ID NO:27) (■, □), SLVIVTTFV (SEQ ID NO:17) (▲ , △) and TLFIGSHVV (SEQ ID NO:24) (●,○). CTL activity was tested on day 6 using a standard 51Cr-release assay.

Figure 12: Prior immunisation of HLA A2/Kb mice with gp85 or gp350 CTL epitopes affords protection against recombinant vaccinia virus challenge. Groups of female A2/Kb transgenic mice, either unimmunized or immunized with CTL epitopes, were challenged with Vacc.gp85 and Vacc.gp350 intraperitonealy. After four days of challenge, these animals were sacrificed and vaccinia titre measured in both ovaries by plaque assay on confluent CV1 cells. X-axis shows peptides used for immunization, while Y-axis shows Mean +/- SE vaccinia virus titre in naive and peptide immunized mice. Recombinant vaccinia virus used for challenge in these animals is shown in each panel of the figure.

DETAILED DESCRIPTION OF THE INVENTION

EXAMPLE 1

Materials and Methods

Establishment and Maintenance of Cell Lines

LCLs were established from sero-positive donors by exogenous virus transformation of peripheral B cells using the B95.8 (Type 1) or Ag876 (Type 2) virus isolates. In addition, LCLs transformed with the B95.8 isolate and expressing different HLA A2 supertypes were also used in this study (12th Histocompatability workshop cell panel; EACC). The peptide transporter (TAP)-negative B x T hybrid cell line 174 x CEM.T2 (referred to as T2) (22)

were used for peptide stablization assays. All cell lines were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin plus 10% foetal calf serum (FCS) (growth medium). Long-term cultures of EBV-negative normal B cell blasts were established as previously described using the CD40 system (referred to as CD40 B cells) (12).

The Burkitt's lymphoma (BL) cell lines, BJAB.gpt1, BJAB.MTLM6, MUTU cl.59 and MUTU cl.216 were used in this study. These were derived from patients with non-endemic or endemic BL. BJAB.MTLM6 and MUTU cl.59 have previously been shown to express LMP1, while BJAB.gpt1 and MUTU cl.216 are negative for LMP1 (5,27). These BL cell lines were routinely maintained in growth medium.

To generate phytohaemagglutinin (PHA) blasts, peripheral blood mononuclear (PBMC) cells were stimulated with PHA (Commonwealth Serum Laboratories, Melbourne) and after 3 days, growth medium containing MLA 144 supernatant and rIL-2 was added (2). PHA blasts were propagated with bi-weekly replacement of IL-2 and MLA supernatant (no further PHA added) for up to 6 weeks.

Virus Isolates

To isolate resident EBV, spontaneous LCLs were established from a panel of 12 unrelated healthy EBV-seropositive donors by spontaneous outgrowth from PBMC cultured in the presence of 0.1μg/ml cyclosporin A (19). In addition, virus isolates from eight different NPC samples (from Southeast Asia) were directly sequenced from biopsy material. These isolates were classified as type 1 EBV based on the DNA sequence divergence within the BAM H1 WYH and E regions of the genome (23,24).

PCR and DNA Sequencing of CTL Epitopes

Specific oligonucleotide primers flanking the DNA region encoding the LMP1 epitope were selected for PCR amplification. The resulting PCR products were purified using QIAquick spin columns (Qiagen Inc. Chatsworth, CA) and sequenced in both directions using a PRISM ready reaction dyedeoxy terminator cycle sequencing kit (Applied Biosystems Inc., Foster City, CA) following the manufacturer's protocol.

Synthesis of Peptides

Peptides synthesized by the Merrifield solid phase method (16) were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in

dimethyl sulphoxide and diluted in serum-free RPMI 1640 medium for use in standard CTL assays.

MHC Stabilisation Assays

To identify the potential HLA A2 binding peptides within LMP1, a computer based program was employed as described elsewhere (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html) (18). These predicted peptides were then used in a standard MHC stablization assay using T2 cells as described earlier (1). Briefly, T2 cells ($2x10^5$) were incubated with 200µl of each of the peptides (200µg/ml) at 26°C for 14-16h, followed by incubation at 37°C for 2-3h. After the incubations, HLA A2 expression was measured by FACS using a monoclonal HLA A2-specific antibody (MA2.1; ATCC).

Generation of Polyclonal and Clonal LMP1-specific CTLs

To generate polyclonal CTLs, PBMC from HLA A2-positive donors were co-cultivated for seven days with the irradiated (8,000 rads) T2 cells sensitized with synthetic peptides. On day 7, these lymphocytes were restimulated with peptide-sensitized T2 cells. After 10 days of culture in growth medium, the cells were used as polyclonal effectors in a standard ^{51}Cr-release assay against peptide-sensitized autologous PHA blasts.

To generate LMP1-specific CTL clones, PBMC (10^6/ml) were cultivated with peptide sensitized autologous lymphocytes (responder to stimulator ratio of 4:1) in 2ml culture wells (Linbro) for 3 days in growth medium. CTL clones, generated by seeding in 0.35% agarose, were established and maintained in growth medium containing highly purified recombinant human IL-2 from E. coli (16), restimulating twice weekly with autologous LCLs. These CTL clones were screened on a panel of recombinant vaccinia-infected autologous CD40 B cells to confirm the antigen specificity (see below).

Vaccinia virus recombinants

Recombinant vaccinia constructs encoding EBV latent antigens and a vaccinia virus construct made by insertion of the pSC11 vector alone and negative for thymidine kinase (Vacc.TK-) have been previously described (6,11). CD40 B cells were infected with recombinant vaccinia virus at a mutiplicity of infection (MOI) of 10:1 for 1 h at 37°C, as described earlier (6,11). After overnight infection, cells were washed with growth medium and processed for CTL assays or for immunoblotting to assess the expression of recombinant EBV antigens (12).

Cytotoxicity Assay

Target cells were either infected with recombinant vaccinia viruses or pre-sensitized with synthetic peptide epitopes (wild-type or variant) and then incubated with ^{51}Cr for 90 min. Following incubation, these cells were washed in growth medium and used as targets in a standard 5 h ^{51}Cr-release assay (16). In some experiments, monoclonal antibodies (MoAb) specific for the non-polymorphic determinants on MHC class I (W6/32) or class II (L243) antigens were added to define the MHC restriction of the CTL clones.

Limiting Dilution Analysis (LDA)

PBMC from HLA A2-positive donors were distributed in graded numbers (two fold dilutions) from 6.25 x 10^3 to 5 x 10^4 cells per well in round-bottomed microtiter plates. Approximately 5 x 10^4 K-irradiated (2,000 rads) peptide sensitized (1µg/ml) auologous PBMC were added to give a total volume of 100 µl. Twenty-four replicates were used at each concentration in each experiment. Cultures were fed on days 4 and 7 with 50 µl of medium supplemented with 20U of rIL-2 and 30% (vol/vol) supernatant from MLA-144 cultures. On day 10, each CTL microculture was split into two replicates and used as effectors in a standard 5 h ^{51}Cr-release assay against autologous PHA blasts precoated with an LMP1 peptide or left uncoated. Wells were scored as positive when the percent specific chromium release for peptide-sensitized target cells exceeded the mean release from untreated control wells by 3 SDs. LDA was performed by the method of maximum likelihood estimation (4). Data from all experiments were compatible with the hypothesis of single-hit kinetics ($P>0.4$) and precursor estimates are given with 95% confidence limits.

Results

Identification of HLA A2 Binding Peptides Within LMP1

To identify potential HLA A2-restricted epitopes within LMP1, the amino acid sequence was analyzed by a computer program designed to predict HLA-binding peptides, based on an estimation of the half-time disassociation of the HLA-peptide complex (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html) (18). A total of 11 peptides with an estimated half-time disassociation score of >400 were selected (Table 1). These peptides were then tested for HLA A2 binding efficiency using HLA A2-positive T2 cells. Representative data from a series of experiments is presented in Fig. 1. This analysis showed that six peptides

significantly increased the expression of HLA A2 on T2 cells suggesting that these peptides bind to this allele.

Generation of Polyclonal CTLs Specific for LMP1 Peptides

The data presented above strongly suggested that LMP1 includes sequences which can bind HLA A2 molecules and are therefore potential targets for virus-specific CTLs. To verify this hypothesis, PBMC from two HLA A2-positive EBV immune donors (SB and AS) were stimulated with T2 cells sensitized with each of the HLA A2-binding peptides from LMP1. On day 10, these effector cells were tested against peptide-sensitized autologous PHA blasts. Represtative data from polyclonal CTLs from donor SB are presented in Fig. 2. Two peptides, YLQQNWWTL (SEQ ID NO:6) and YLLEMLWRL (SEQ ID NO:1), showed significant activation of polyclonal CTLs; however, the YLLEMLWRL-stimulated CTLs consistently showed significantly stronger CTL activity when compared to the YLQQNWWTL-stimulated cells. Similar data were also obtained for another HLA A2-positive donor (AS) (data not shown).

Table 1: ***Identification of Potential HLA A2 Binding Peptides within LMP1****

Ranking	Residue	Peptide Sequence	Score (Estimate half-time disassociation from HLA A2 allele)
1	125-133	YLLEMLWRL (SEQ ID NO:1)	25714.76
2	32-40	LLLALLFWL (SEQ ID NO:2)	9858.69
3	167-175	LLVDLLWLL (SEQ ID NO:3)	2568.45
4	92-100	LLLIALWNL (SEQ ID NO:4)	2317.87
5	173-181	WLLLFLAIL (SEQ ID NO:5)	1302.88
6	159-167	YLQQNWWTL (SEQ ID NO:6)	1252.90
7	166-174	TLLVDLLWL (SEQ ID NO:7)	999.86
8	171-179	LLWLLLFLA (SEQ ID NO:8)	935.11
9	152-160	ILLIIALYL (SEQ ID NO:9)	739.03
10	110-118	VLFIFGCLL (SEQ ID NO:10)	510.60
11	132-140	RLGATIWQL (SEQ ID NO:11)	441.60

*To identify the potential HLA A2 binding peptides within LMP1, a computer based program was employed as described elsewhere (18).
This program can be directly accessed through the world-wide web site:
http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html

Charaterization of LMP1 CTL epitope

To further characterize the CTL epitopes identified by the polyclonal CTLs, virus-specific CTL clones were generated using initial stimulation with peptide sensitized autologous PBMC followed by continous restimulation with irradiated autologous LCLs. Proliferating clones were screened for peptide recognition using the rapid visual assay for CTL specificity (2) and one clone, SB7, clearly recognized the YLLEMLWRL (SEQ ID NO:1) peptide. No CTL clones specific for YLQQNWWTL (SEQ ID NO:6) were isolated using this procedure. To further confirm the antigen specificity of the SB7 clone, autologous CD40-stimulated B cells were infected with recombinant vaccinia viruses encoding individual EBV antigens and then exposed to these CTLs. The data presented in Fig. 3A clearly demonstrate that only target cells infected with the LMP1-expressing vaccinia construct were recognized. In addition, only LMP1- and HLA A2-positive BL cell lines (BJAB.MTLM6 or Mutu cl.59) were efficiently lysed by this clone, while BL cells negative for this antigen (BJAB.gpt1 and Mutu cl.216) were not recognized (Fig. 3B). These results confirm that YLLEMLWRL (SEQ ID NO:1) is an LMP1 CTL epitope that is endogenously processed by virus-infected cells.

In the next set of experiments we analyzed the frequency of CTL precursors (CTLp) for this epitope in PBMC from HLA A2-positive healthy EBV immune donors by limiting dilution analysis. YLLEMLWRL-specific CTLp were reactivated in vitro by stimulation of PBMCs from donors SB and AS with autologous peptide-sensitized PBMCs. Autologous PHA blasts precoated with peptide YLLEMLWRL (SEQ ID NO:1) or untreated were used as target cells in a chromium release assay. The representative data in Fig. 4 show that a very low frequency of memory CTL specific for the YLLEMLWRL (SEQ ID NO:1) epitope were detected in the HLA A2-positive donors AS (1:223,535 +/- 107,437) and SB (1:252,650 +/- 122,875).

To determine whether EBV-transformed LCLs expressing different HLA A2 supertypes could be recognized by clone SB7, a panel of LCLs expressing ten different supertypes of the HLA A2 allele (HLA A*0201-HLA A*0210) were screened in a standard CTL assay. The data presented in Fig. 5 clearly demonstrate that LCLs expressing all the major HLA A2 supertypes except HLA A*0205 were efficiently recognized by the CTL clone SB7. This lysis was significantly inhibited by the HLA class I-specific antibody W6/32.

Surprisingly, HLA A2-positive and TAP-negative T2 cells were also recognized by this clone suggesting that the YLLEMLWRL (SEQ ID NO:1) epitope is endogenously processed through a TAP-independent pathway (Fig. 5).

Sequence Analysis of the HLA A2-Restricted LMP1 Epitope in Virus Isolates from NPC Patients and Healthy Donors

Efficient presentation of the LMP1 epitope by HLA A*0201, HLA A*0203 and HLA A*0207, which are common supertypes in the Southeast Asian ethnic population, raised the possibility that this epitope might be important as a potential target epitope for LMP1-expressing NPC. Sequence analysis across this CTL epitope region in virus isolates from eight NPC samples was carried out using LMP1-specific primers. Spontaneous LCLs from healthy EBV immune donors were used as controls in this analysis. Interestingly all EBV isolates from the NPC samples displayed identical substitutions within this epitope (Table 2).

In contrast, of the 12 virus isolates from healthy EBV immune donors, four encoded a sequence identical to that of the B95.8 isolate, while six displayed a different pattern of alterations within this epitope that differed from those found in the NPC samples. In some isolates leucine at position 2, methionine at position 5 and arginine at position 8 were substituted with phenylalanine, isoleucine and glycine, respectively (Table 2), while in other isolates only the methionine at position 5 was substituted with isoleucine. Importantly, incubation of T2 cells with these variant peptides (YFLEILWGL (SEQ ID NO:32) and YLLEILWRL (SEQ ID NO:33)) significantly increased MHC expression on these cells (Fig. 6A), indicating efficient binding to HLA A2. the YLLEILWRL (SEQ ID NO:33) peptide was efficiently recognized by the SB7 clone, while no CTL activity was seen in the presence of the YFLEILWGL (SEQ ID NO:32) peptide (Fig. 6B). The latter result does not rule out the possibility that YFLEILWGL (SEQ ID NO:32) is an epitope individuals infected with an EBV strain encoding this sequence. The loss of recognition by the clone SB7 is likely to be due an inappropriate T cell receptor interaction with the MHC-peptide complex rather than loss of MHC binding. Thus it is possible that T cells expressing a different T cell receptors are capable of efficiently recognising this HLA binding peptide (8). Interestingly, virus isolates from the other two healthy EBV immune donors

Table 2: Sequence of HLA A2-restricted LMP1 epitope (YLLEMLWRL) in EBV isolates from NPC and healthy seropositive individuals.

Virus	Origin	Epitope Sequence[a]	HLA A2[b] Binding	Number of Isolates
B95.8		[96676]TAC TTA TTG GAG ATG CTC TGG CGA CTT[96702] Y L L E M L W R L	++++	
NPC	Southeast Asia	---- --C ---- ---- --T ---- ---- ---- ---- - F - - I - - - -	-	8
Healthy donors	Caucasian	---- ---- ---- ---- ---- ---- ---- ---- ---- - - - - - - - - -	++++	4
	Caucasian	---- ---- ---- ---- --T ---- ---- ---- ---- - - - - I - - - -	++++	2
	Caucasian	---- --C ---- ---- --T ---- ---- G-G ---- - F - - I - - G -	++++	4
	Caucasian	---- --C ---- ---- --T ---- ---- ---- ---- - F - - I - - - -	-	1
	Southeast Asia	---- --C ---- ---- --T ---- ---- ---- ---- - F - - I - - - -	-	1

[a] Oligonucleotide primers used for PCR amplification were (YLL1:CTTCGGGTGCTTACTTGGTA; YLL2:TCATCGTGGTGGTGTTCA 3')

[b] HLA A2 binding data is adapted from Fig. 6. ++++ indicates strong binding; - indicates no binding

(including one from Southeast Asia) displayed changes within the epitope sequence which were identical to those seen in the NPC samples (Table 2).

Analysis of Antigen Processing Function of NPC cells

To determine whether NPC cells can present endogenously expressed antigens to virus-specific CTLs, tumour cells were either infected with recombinant vaccinia encoding EBNA4 (Vacc.EBNA4) or presensitized with synthetic peptide epitopes. Figure 7 illustrates the results from an experiment in which recombinant vaccinia-infected NPC cells (C15) were compared with HLA-matched type 2 LCLs infected with Vacc.EBNA4. Following exposure to EBNA4-specific CTLs, Vacc.EBNA4-infected or peptide sensitized NPC cells were efficiently recognised by both CM9 and CM29 CTL clones. The level of CTL lysis was comparable to that seen for LCLs. These results clearly demonstrate that NPC cells are able to transport sufficient levels of peptides into the ER by TAP-dependent mechanism and can efficiently transport MHC-peptide complexes from the ER to the surface of the cell.

Normal antigen processing function in NPC cells has significant implications for vaccines designed to control these tumours in *vivo*. Earlier studies have demonstrated that the latent gene expression in NPC is often limited to EBNA1 and the transmembrane proteins, LMP1 and LMP2 (29).

Since EBNA1 is not recognized by EBV-specific CTLs, there is an increasing emphasis on designing strategies to control NPC around epitopes known to be included within LMP1 (6,17). In view of the data presented in this study, it is reasonable to assume that LMP epitopes will be processed efficiently by NPC cells. An effective approach to control NPC cells in vivo may be to amplify LMP-specific CTL responses in these patients. This might be achieved by two different procedures. Firstly, NPC patients might be immunised with synthetic peptides which include CTL epitopes from LMP1 and/or LMP2. Alternatively, LMP1 and LMP2-specific CTLs from HLA matched healthy virus carriers may be adoptively transferred into NPC patients in a manner analogous to that used to successfully treat EBV-associated polyclonal lymphomas in bone marrow transplant recipients (30).

Discussion

Earlier work from various laboratories have shown that the viral phenotype of EBV-associated malignancies is likely to be a very important factor in reducing tumor susceptibility to virus-specific CTL surveillance,

since viral antigen expression in these malignant cells in vivo is restricted to either EBNA1, or EBNA1 and LMP1 (9,20,21). Since it is now firmly established that EBNA1 does not include class I-restricted CTL epitopes (6,17), considerable interest has been directed towards identifying potential epitopes within LMP1. The present study was precisely designed to address this issue. One of the limiting factors in identifying epitopes within LMP1 has been the fact that the CTL response to this antigen often constitutes as a minor component of the total virus-specific response (6,17). To overcome this problem we employed a modified protocol to identify potential HLA A2-restricted epitopes within LMP1. An important step in this process was the use of a computer based program developed by Parker and colleagues (18) designed to predict the potential HLA binding peptides within various proteins from human pathogens. Analysis of the LMP1 sequence from the B95.8 EBV isolate revealed a number of potential HLA A2 binding peptides and a large proportion of these were then functionally shown to stabilize HLA A2 molecules on T2 cells. Stimulation of PBMCs with these peptides resulted in the activation of a strong polyclonal CTL response specific for the peptide YLLEMLWRL (SEQ ID NO:1), while a weaker response was seen for another peptide YLQQNWWTL (SEQ ID NO:6). The YLLEMLWRL (SEQ ID NO:1) sequence was confirmed as an LMP1 epitope by isolating a CTL clone (SB7) specific for this peptide. The LMP1 specificity of the SB7 CTL clone was further confirmed by the recombinant vaccinia experiments and efficient lysis of LMP1 expressing HLA A2-positive BL cells.

The CTL response characterized in the present report is of interest not only because it is directed against a viral antigen constitutively expressed in many EBV-associated malignancies (HD and NPC) but also because the HLA A2 allele is common in virtually all human populations (13). More importantly, EBV transformed LCLs expressing all the major HLA A2 supertypes were efficiently recognized by the LMP1-specific CTL clone, a result that has important implications for anti-viral vaccine design aimed at protect different ethnic populations. It is important to mention here that the CTL response to the LMP1 epitope in healthy seropositive individuals constitutes a minor component of the virus-specific CTL response and very low levels of CTL precursors are seen for this epitope. It may nevertheless be possible to amplify this component by vaccination with the relevant peptide

or with adoptive transfer of in vitro activated LMP1-specific CTLs. Such approaches may be of use in the control of HD and NPC.

Efficient presentation of the YLLEMLWRL (SEQ ID NO:1) peptide by HLA A*0201, HLA A*0203 and HLA A*0207-positive LCLs, which are common supertypes in the southeast Asian population, raises the possibility that this epitope might be exploited as a potential target epitope for LMP1-expressing NPC.

EXAMPLE 2

Materials and Methods

Infectious Mononcleosis (IM) Patients

IM patients, identified on clinical grounds and by heterophile antibody positivity, were bled during the first 5-10 days of illness and, in two cases, on a second occasion 24-36 months after the resolution of symptoms. These patients were HLA typed for the HLA A2 allele by serotyping in microcytotoxicity and by genotyping. Three patients (SB, LP and MG) were identified as HLA A2-positive patients and this was subsequently confirmed by FACS analysis using an HLA A2-specific monoclonal antibody (ATCC).

Establishment and Maintenance of Cell Lines:

EBV-transformed lymphoblastoid cell lines (LCLs) were established from a panel of IM and healthy EBV-seropositive donors by exogenous virus transformation of peripheral B cells using type 1 (B95.8) or type 2 (Ag876) EBV isolates (16), and were routinely maintained in RPMI 1640 containing 2 mM glutamine, 100 IU/ml penicillin and 100 ug/ml streptomycin plus 10% foetal calf serum (FCS) (growth medium). In addition, the peptide transporter (TAP)-negative B x T hybrid cell line 174 x CEM.T2 (referred to as T2) (22) were used for peptide stablization assays.

To generate phytohaemagglutinin (PHA) blasts, peripheral blood mononuclear cells (PBMC) were stimulated with PHA (Commonwealth Serum Laboratories, Melbourne) and after 3 days, growth medium containing MLA 144 supernatant and rIL-2 was added (36). PHA blasts were propagated with bi-weekly replacement of IL-2 and MLA supernatant (no further PHA added) for up to 6 weeks.

Establishment and Preparation of CTL Effectors

Acute IM PBMC effectors for use in ex vivo cytotoxicity assays were resuspended in growth medium supplemented with recombinant IL2 and used directly in a cytotoxicity assay (see below). To generate polyclonal

CTLs. PBMCs from HLA A2-positive donors were co-cultivated for seven days with irradiated (8,000 rads) T2 cells presensitized with synthetic peptides (37). On days 7 and 14, these cultures were restimulated with peptide-sensitized T2 cells. After 18 days of culture in growth medium, the cells were used as polyclonal effectors in a standard 51Cr-release assay against peptide-sensitized autologous PHA blasts.

Synthesis of Peptides

Peptides, synthesized by the Merrifield solid phase method, were purchased from Chiron Mimotopes (Melbourne, Australia), dissolved in dimethyl sulphoxide, and diluted in serum-free RPMI 1640 medium for use in standard CTL assays.

MHC Stabilisation Assays

HLA A2 binding peptides within the gp85 and gp350 antigens were identified using a protocol as described in Example 1. These predicted peptides were then used in a standard MHC stablization assay using T2 cells. Briefly, T2 cells ($2x10^5$) were incubated with 200µl of each of the peptides (200 µg/ml) at 26°C for 14-16h, followed by incubation at 37°C for 2-3h. After the incubations, HLA A2 expression was measured by FACS using a monoclonal HLA A2-specific antibody (MA2.1; ATCC).

Vaccinia Virus Recombinants

Recombinant vaccinia constructs encoding the EBV structural antigens gp350 (Vacc.gp350) and gp85 (Vacc.gp85), and a vaccinia virus construct made by insertion of the pSC11 vector alone and negative for thymidine kinase (Vacc.TK-) have been previously described (38). Target cells were infected with recombinant vaccinia virus at a mutiplicity of infection (MOI) of 10:1 for 1 h at 37°C, as described earlier (6,12). After overnight infection, cells were washed with growth medium and processed for CTL assays or for immunoblotting to assess the expression of recombinant EBV antigens (11).

Cytotoxicity Assay

Target cells were either infected with recombinant vaccinia viruses or pre-sensitized with synthetic peptide epitopes and then incubated with 51Cr for 90 min. Following incubation, these cells were washed in growth medium and used as targets in a standard 5 h 51Cr-release assay (16).

Immunisation of HLA A2/Kb transgenic mice with gp350 and gp85 CTL epitopes

HLA A2/K b transgenic mice used in this study have been described elsewhere (39). These mice express a chimeric class I molecule composed of the alpha 1 & 2 domains of the human A*0201 allele and the alpha 3 domains of the mouse H-2Kb class I molecules. Peptide immunizations were carried out as described by Vitello and colleagues (40). Briefy, these animals were twice immunized (at a 14 day interval) subcutaneously with 50ug/mouse of CTL epitopes emulsified in IFA together with 5ug of Tetanus Toxoid as a source of help. Four weeks following peptide immunization, animals were assessed for gp350- and gp85-specific CTL response. For assessing these CTL responses, splenocytes ($3x10^6$ cells/ml) were cocultured with syngeneic, irradiated (2000 rad) peptide-coated LPS blasts ($3x10^5$ cells/ml) and 3ug/ml human B2-microglobulin. CTL activity was tested on day 6 using a standard 51Cr-release assay.

Vaccinia Protection Assay

For protection experiments, groups of 8 weeks old female A2/Kb transgenic mice were immunized with CTL epitopes as described above. On day 28, mice were challenged with Vacc.gp85 and Vacc.gp350 intraperitonealy ($1x10^7$ pfu in 100ul PBS). After four days of challenge, these animals were sacrificed and vaccinia titres measured in both ovaries by plaque assay on confluent CV1 cells.

Results

Identification of HLA A2 Binding Peptides Within gp85 and gp350

To identify potential HLA A2-restricted epitopes within gp85 and gp350, the amino acid sequence was analyzed by a computer program designed to predict HLA-binding peptides, based on an estimation of the half-time disassociation of the HLA-peptide complex (http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html) (18). A total of 20 peptides (13 from gp85 and 7 from gp350) with an estimated half-time disassociation score of >100 for gp85 and >50 for gp350 were selected (Table 3). These peptides were then tested for HLA A2 binding efficiency using HLA A2-positive T2 cells. Representative data from a series of experiments is presented in Figure 8. This analysis showed that seven of these peptides significantly increased the expression of HLA A2 on T2 cells

Table 3: ***Identification of Potential HLA A2 Binding Peptides within gp85 and gp350****

Ranking	Residue	Peptide Sequence	Score (Estimate half-time disassociation from HLA A2 allele)
gp85 peptides			
1	177-185	FLMGTYKRV (SEQ ID NO:12)	1775.663
2	317-325	WLAKSFFEL (SEQ ID NO:13)	1082.903
3	672-680	GLYEERAHV (SEQ ID NO:14)	912.522
4	685-693	ILYFIAFAL (SEQ ID NO:15)	674.026
5	2-10	QLLCVFCLV (SEQ ID NO:16)	488.951
6	225-233	SLVIVTTFV (SEQ ID NO:17)	382.536
7	681-689	VLAIILYFI (SEQ ID NO:18)	224.537
8	684-692	IILYFIAFA (SEQ ID NO:19)	196.407
9	542-550	LMIIPLINV (SEQ ID NO:20)	181.738
10	1-9	MQLLCVFCL (SEQ ID NO:21)	181.738
11	7-15	FCLVLLWEV (SEQ ID NO:22)	133.298
12	658-666	YLLLTTNGT (SEQ ID NO:23)	126.883
13	420-428	TLFIGSHVV (SEQ ID NO:24)	105.510
gp350 peptides			
1	871-879	VLTLLLLLV (SEQ ID NO:25)	271.948
2	152-160	LIPETVPYI (SEQ ID NO:26)	126.481
3	863-871	VLQWASLAV (SEQ ID NO:27)	118.238
4	875-883	LLLLVMADC (SEQ ID NO:28)	71.872
5	67-75	QLTPHTKAV (SEQ ID NO:29)	69.552
6	861-869	MLVLQWASL (SEQ ID NO:30)	61.737
7	873-881	TLLLLLVMA (SEQ ID NO:31)	42.278

*To identify the potential HLA A2 binding peptides within gp85 and gp350, a computer based program was employed as described elsewhere (ref 18).

This program can be directly accessed through the world-wide web site: http://bimas.dcrt.nih.gov/molbio/hla_bind/index.html

suggesting that these peptides might be potential HLA A2-restricted epitopes.

Recognition of the gp85 and gp350 Peptide Epitopes by IM Effectors Ex Vivo

The seven HLA A2-binding peptides, which included four peptides from gp85 and three peptides from gp350 were next tested for CTL recogniton by effectors from IM patients. In addition, we also included an HLA A2-restricted CTL epitope from EBV latent mambrane protein (LMP1) as a positive control (38). PBMCs from three HLA A2-positive IM patients, SB, LP and MG were resuspended in IL2-supplemented growth medium and used as effectors in a standard 51Cr-release assay against HLA-matched PHA blasts sensitized with the gp85, gp350 or LMP1 peptides. Representative data from two different experiments is shown in Figure 9(A-C). Effectors from all three IM patients showed clear recognition of the reference LMP1 peptide (YLQQNWWTL (SEQ ID NO:6)) consistent with our earlier finding that this peptide is recognized by EBV-specific CTLs. More importantly, these IM patients also showed strong recognition of target cells sensitized with selected gp85 or gp350 peptides. Interestingly, each of these individuals showed a distinct pattern of reactivity against these peptides. IM patient SB showed strong reactivity against peptides SLVIVTTFV (SEQ ID NO:17) (gp85) and VLQWASLAV (SEQ ID NO:27) (gp350) (Figure 9A), while the LP and MG effectors recognised target cells preloaded with peptides LMIIPLINV (SEQ ID NO:20) (gp85) and VLQWASLAV (SEQ ID NO:27) (gp350) (Figure 9(B-C)). Furthermore, ex vivo effectors from patient LP also recognised target cells infected with Vacc.gp350 and Vacc.gp85 (Figure 9D).

In Vitro Expansion of gp85 and gp350 Peptide Epitope Reactive CTLs

The data presented above clearly demonstrate that gp85 and gp350 include CTL determinants which can bind HLA A2 molecules and are efficiently recognised by ex vivo effectors from IM patients. To determine whether gp85- or gp350-reactive CTLs can be detected following recovery from IM, PBMCs from the two donors SB and LP were collected at 24-36 months post IM respectively and were stimulated with T2 cells presensitized with each of the gp85 and gp350 peptides which showed strong HLA A2 binding. On day 18, these CTL effector were tested against peptide-sensitized autologous PHA blasts. Representative data from polyclonal CTLs from donor SB are presented in Figure 10. CTL effectors from donor SB not

only showed strong reactivity against peptides SLVIVTTFV (SEQ ID NO:17) and VLQWASLAV (SEQ ID NO:27) but also recognized two other peptides from gp85 (LMIIPLINV (SEQ ID NO:20) and TLFIGSHVV (SEQ ID NO:24)). Donor LP also showed a similar pattern of CTL lysis. Thus peptide TLFIGSHVV (SEQ ID NO:24) was a target for EBV-specific CTL recogntion in the memory response of these A2-positive individuals, but this response was not detectable with ex vivo effectors during acute infection. Another important point which needs to be highlighted here is that our attempts to activate gp85- or gp350-specific CTLs with autologus LCLs as stimulators were unsuccessful. This result is not surprising since it is well established that in latently infected B cells, gp350 or gp85 antigens are poorly expressed. The LCL-stimulated polyclonal T cell lines from these donors strongly reactive against latent antigens (data not shown). This obseravtion is consistent with our earlier studies which showed that CTL responses in healthy virus carriers is often dominated by reactivity to latent antigens (6). Another explanation for an inability to detect gp350- or gp85-specific CTL reactivity following stimulation with the autologous LCLs is that these responses may constitute a minor component of the total virus specific CTL respouse in healthy virus carriers. Indeed, limiting dilution analysis for CTL precursors specific for the gp350 or gp85 peptide epitopes in post IM donors SB and LP showed precursor frequencies of >1/50,000, while precursor frequencies for CTLs that recognise CTL epitopes within the latent antigens were between 1/4,000-1/15,000 (data not shown).

Immunization of HLA A2/Kb Mice with gp85 and gp350 Peptide Epitopes Induces Specific CTL Response

Having established that gp85 and gp350 includes CTL epitopes, we extended our studies to explore the possibility of using these peptide epitopes to induce specific CTL response in vivo. HLA A2/Kb transgenic mice were used as an experimental model to address this issue. These mice express a chimeric class I molecule composed of the alpha 1 & 2 domains of the human A*0201 allele and the alpha 3 domain of the mouse H-2Kb class I molecules. These animals were immunized subcutaneously with gp350 or gp85 CTL epitopes emulsified in IFA together with Tetanus Toxoid as a source of help. The SLVIVTTFV (SEQ ID NO:17) and TLFIGSHVV (SEQ ID NO:24) peptides from gp85 and the VLQWASLAV (SEQ ID NO:27) peptide from gp350 were used for immunisation. Two weeks following

immunization, specific CTL response was assessed in each mouse using splenocytes or pooled inguinal lymph node cells as effectors. Data presented in Figure 11(A-C) demonstrate that peptide epitopes from gp85 (SLVIVTTFV (SEQ ID NO:17) and TLFIGSHVV (SEQ ID NO:24)) and gp350 (VLQWASLAV (SEQ ID NO:27)) induced strong CTL response in splenocytes. Interestingly, CTLs activated from splenocytes with peptide TLFIGSHVV (SEQ ID NO:24) consistently showed strong lysis of targets, while splenocytes from SLVIVTTFV (SEQ ID NO:17) and VLQWASLAV (SEQ ID NO:27) immunized mice showed variable in vitro CTL lysis. A strong specific CTL activity was also noticed in pooled lymphocytes from inguinal lymph nodes (Figure 11D).

Prior Immunisation of HLA A2/Kb mice with gp85 or gp350 CTL Epitopes Affords Protection Against Recombinant Vaccinia Virus Challenge

Four weeks after peptide immunization with gp85 or gp350 CTL epitopes, HLA A2/K b mice were challenged with 10^7 pfu of recombinant vaccinia virus encoding either gp85 or gp350. After four days of challenge, these animals were sacrificed and vaccinia titres measured in both ovaries by plaque assay on confluent CV1 cells. Data from one such experiment is presented in Figure 12. Animals immunised with gp85 and gp350 epitopes showed very low to undetectable virus in their ovaries, while in naive mice very high titres of vaccinia virus were detected. This protection correlated with strong induction of epitope-specific CTL responses detected in the splenocytes and lymph node cells collected four weeks after primary peptide vacciniation in HLA A2/Kb transgenic mice.

Discussion

There is increasing interest in formulating an effective vaccine against EBV, designed to not only limit the outgrowth of latently infected B cells in healthy individuals but to also block the development of many EBV-associated malignancies such as Burkitt's lymphoma (BL), nasopharyngeal carcinoma (NPC) and Hodgkin's disease (HD). In western societies, the principle aim of such a vaccine would be to protect from IM. In this context, virus load (a large dose of orally transmitted virus and/or overexpansion of the virus-transformed B cell pool beyond a critical threshold) may be a critical determinant of disease risk (7). Therefore, a vaccine capable of either blocking primary EBV infection or significantly reducing the EBV load during primary infection may be adequate to avert clinical symptoms. A similar vaccine will also be able to reduce the immediate risk of

lymphoproliferative disease in transplant patients receiving immunosuppressive therapy. On the other hand, EBV-associated malignancies such as BL, NPC, and HD arise in patients years after their primary infection, and protection from these longer-term consequences would require a vaccine that ideally confers sterile immunity and prevents the establishment of the carrier state.

EBV structural antigens, primarily gp350, have long been considered as the potential candidates for an EBV vaccine. The suggestion that gp350 is a likely vaccine candidate was based initially upon the observation that this glycoprotein is the principal target of the virus-neutralizing antibody response (41). A number of recombinant formulations of gp350, either presented as a subunit antigen or expressed from recombinant viral vectors, designed to induce high titre neutralizing antibodies, have shown significant protection against EBV-induced B cell lymphomas in cotton-top tamarins (31). However, development of neutralizing antibodies in vaccinated animals does not always shows limited correlation with protection from EBV infection, although recent results have suggested a role for gp350-specific CTLs in this protection (34). If the latter suggestion is correct, it is important to identify the potential CTL determinants within EBV structural proteins since it is now well established that immunization with whole viral proteins is unable to elicit an efficient CTL response. Moreover, a vaccine based on CTL epitopes provides an opportunity to include determinants not only from gp350 but also from other structural antigens, such as gp85. To address this issue we have used a novel protocol to successfully identify CTL epitopes within gp350 and gp85. In the first set of experiments we identified HLA A2 binding peptides within gp350 and gp85. Subsequent experiments were focussed on IM patients with the HLA A2 allele. Using ex vivo primary effectors, we observed strong reactivity to three different gp350 and gp85 peptides. Interestingly, individual IM patients showed distinct patterns of reactivity to each of these peptides. Strong reactivity against peptides SLVIVTTFV (SEQ ID NO:17) (gp85) and VLQWASLAV (SEQ ID NO:27) (gp350) was observed with ex vivo effectors from patient SB, while the LP and MG effectors recognized target cells preloaded with LMIIPLINV (SEQ ID NO:20) (gp85) and VLQWASLAV (SEQ ID NO:27) (gp350) peptides. More importantly, ex vivo effectors from patient LP also recognised target cells infected with Vacc.gp350 and Vacc.gp85. Interestingly, the level of ex vivo

CTL lysis directed to epitopes from structural antigens was consistently higher than those seen in the same assays against HLA A2-restricted CTL epitopes from a latent antigen. These results are consistent with recent observations by Steven and colleagues (42) that ex vivo CTL reactivity to lytic antigens in IM patients is significantly higher compared to latent antigens.

In the next set of experiments, we explored the possibility of detecting structural antigen-specific CTL responses in individuals following resolution of IM symptoms. This follow up analysis was carried out 24-36 months post acute IM. Our initial attempts to isolate gp350- or gp85-specific CTLs from post IM donors by stimulating with the autologous LCL were unsuccessful. Subsequently we used peptide loaded T2 cells as stimulators to generate gp350- and gp85-specific CTLs. We have recently shown that this method can be successfully used to raise low frequency EBV-specific CTL precursors (38). Stimulation of PBMC from donors SB and LP raised strong CTL responses to the gp85 and gp350 CTL epitopes. Both donors SB and LP not only showed reactivity against peptides SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20) and VLQWASLAV (SEQ ID NO:27) but also recognized another peptide from gp85, TLFIGSHVV (SEQ ID NO:24). It is interesting to note here that both donors showed no ex vivo CTL reactivity to TLFIGSHVV (SEQ ID NO:24) during acute IM. One of the important conclusions drawn from these analyses is that, following recovery from acute IM, there is a significant reduction in CTL precursors to the structural antigens, and the response becomes dominated by CTL reactive to the latent antigens. Indeed, limiting dilution analysis for CTL precursors specific for the gp350 or gp85 peptide epitopes in donors SB and LP post IM showed frequencies of >1/50,000, while precursor frequencies for CTL epitopes within latent antigens were between 1/4,000-1/15,000.

The detection of a strong ex vivo CTL response in IM patients to the structural antigens has important implications for any future vaccine design. As mentioned above, to date, the major emphasis of vaccine design based on EBV structural antigens has been directed towards generating a strong neutralizing antibody response. However, these neutralising antibody response fail to correlate with protection against EBV-induced polyclonal lymphomas in cotton-top marmosets. Nevertheless, it is possible that this protection is mediated by structural antigen-specific CTL responses. To

address this issue, we employed an experimental animal model system to determine whether gp350 or gp85 CTL epitope immunized transgenic mice, expressing the human HLA A2 antigen, are capable of (a) generating structural antigen-specific CTL responses and (b) reducing infection with a recombinant vaccinia virus infection expressing the gp350 or gp85 antigen. These mice not only showed induction of a strong CTL response following immunization but also acquired strong resistance to virus infection. It is important to mention here that although this experiment does not allow any firm conclusions on the efficacy of a gp350 and/or gp85 CTL epitope based vaccine in humans, it does clearly show that CTL epitopes from the EBV structural antigens can be used as immunogens to induce an efficient CTL response in vivo. Moreover, this approach also overcomes limitations of whole gp350 or gp85 proteins which might be inefficient at eliciting CTL responses in humans. Obviously one of the possible obstacles of any epitope-based approach to vaccination in humans is HLA polymorphism because epitope choice is allele-specific. However, this obstacle might be overcome using appropriate mixtures of synthetic peptide epitope or by constructing vectors to express polypeptides in which the relevant epitope sequences are linearly joined together. Indeed, earlier studies from our laboratory have shown that if such an EBV polyepitope sequence is expressed within cells from a recombinant vaccinia vector, all of the constituent epitopes are efficiently presented for CTL recognition (43), indicating the potential of this approach as a vaccine strategy. More recently, work in a murine model has also shown that each of several CTL epitopes combined in a polyepitope construct was capable of eliciting a CTL response in vivo and could protect the animals from subsequent challenge (44). In the long term, it may be possible to combine CTL epitopes from the EBV structural antigens with latent antigen epitopes generating a chimeric protein that fuses the important immunogenic determinants from the two different types of antigens to design an effective vaccine.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

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Claims:

1. A cytotoxic Epstein-Barr virus (EBV) T-cell epitope, the epitope being derived from an EBV structural antigen.

2. A cytotoxic EBV T-cell epitope as claimed in claim 1 wherein the EBV structural antigen is gp85 or gp350.

3. A cytotoxic T-cell epitope, the epitope being selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

4. A subunit vaccine including a cytotoxic Epstein-Barr virus (EBV) T-cell epitope as claimed in claim 1 or claim 2.

5. A subunit vaccine including at least one T-cell epitope selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

6. A subunit vaccine as claimed in claim 5 wherein the epitope is selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YLQQNWWTL (SEQ ID NO:6), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24),and VLQWASLAV(SEQ ID NO:27).

7. A subunit vaccine as claimed in any one of claims 4 to 6 wherein the vaccine further includes at least one antigen to which the individual will

mount an anamnestic response in addition to the at least one cytotoxic T-cell epitope.

8. A subunit vaccine as claimed in claim 7 wherein the at least one antigen is selected from the group consisting of tetanus toxoid, diphtheria toxoid, Bordetella pertussis antigens, poliovirus antigens, purified protein derivative (PPD), gp350 protein, helper epitopes and combinations thereof.

9. A subunit vaccine as claimed in claim 8 wherein the at least one antigen is tetanus toxoid.

10. A subunit vaccine as claimed in any one of claims 4 to 9 in which the vaccine includes a water-in-oil formulation.

11. An isolated nucleic acid sequence encoding a cytotoxic Epstein-Barr virus (EBV) T-cell epitope as claimed in claim 1 or claim 2.

12. An isolated nucleic acid sequence encoding at least one of the cytotoxic T-cell epitopes selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29).

13. An isolated nucleic acid sequence as claimed in claim 12 wherein the epitope is selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YLQQNWWTL (SEQ ID NO:6), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24),and VLQWASLAV (SEQ ID NO:27).

14. A vector including a nucleic acid sequence as claimed in any one of claims 11 to 13.

15. A vector as claimed in claim 14 in which the vector is a bacteria, preferably *Salmonella spp.*

16. A vector as claimed in claim 14 in which the vector is a virus, preferably Adenovirus, Retrovirus or Vaccinia, and most preferably Modified Vaccinia Ankara.

17. An isolated polypeptide, the polypeptide including at least one EBV CTL epitope as claimed in any one of claims 1 to 3.

18. A method of preparing a composition for use in inducing CTLs in a subject, the method including admixing at least one epitope as claimed in any one of claims 1 to 3 with at least one pharmaceutically acceptable carrier, diluent or excipient.

19. A method of reducing the risk of EBV infection in a subject which method includes administering to the subject an effective amount of:-

(1) at least one CTL epitope as claimed in any one of claims 1 to 3;

(2) a subunit vaccine as claimed in any one of claims 4 to 10;

(3) a nucleic acid sequence as claimed in any one of claims 11 to 13;

(4) a vector as claimed in any one of claims 14 to 16;

or

(5) a polypeptide as claimed in claim 17.

20. A method of treating or preventing nasopharyngeal carcinoma or Hodgkin's disease in a subject which method includes administering to the subject an effective amount of at least one CTL epitope derived from an EBV structural or latent antigen.

21. A method according to claim 20 wherein the EBV structural antigen is gp85 or gp350.

22. A method according to claim 20 wherein the EBV latent antigen is LMP1 or LMP2.

23. A method according to claim 20 wherein at least one CTL epitope selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL(SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29), is administered to the subject.

24. A method of treating or preventing growth of NPC cells in a subject in need thereof which method includes administering to the subject at least one CTL epitope derived from an EBV structural or latent antigen.

25. A method according to claim 24 wherein the EBV structural antigen is gp85 or gp350.

26. A method according to claim 24 wherein the EBV latent antigen is LMP1 or LMP2.

27. A method according to claim 27 wherein at least one CTL epitope selected from the group consisting of YLLEMLWRL (SEQ ID NO:1), YFLEILWGL (SEQ ID NO:32), YLLEILWRL (SEQ ID NO:33), YLQQNWWTL (SEQ ID NO:6), LLLALLFWL (SEQ ID NO:2), LLVDLLWLL (SEQ ID NO:3), LLLIALWNL (SEQ ID NO:4), WLLLFLAIL (SEQ ID NO:5), TLLVDLLWL (SEQ ID NO:7), LLWLLLFLA (SEQ ID NO:8), ILLIIALYL (SEQ ID NO:9), VLFIFGCLL (SEQ ID NO:10), RLGATIWQL (SEQ ID NO:11), ILYFIAFAL (SEQ ID NO:15), SLVIVTTFV (SEQ ID NO:17), LMIIPLINV (SEQ ID NO:20), TLFIGSHVV (SEQ ID NO:24), LIPETVPYI (SEQ ID NO:26), VLQWASLAV (SEQ ID NO:27) and QLTPHTKAV (SEQ ID NO:29), is administered to the subject.

28. A method of treating or preventing the growth of NPC or HD cells in a first subject which method includes transferring to the first subject EBV-specific CTLs which recognise NPC or HD cells.

29. A method as claimed in claim 28 wherein the EBV-specific CTLs are obtained from the first subject by *in vitro* stimulation of CTLs by exposure to EBV CTL epitopes.

30. A method as claimed in claim 28 wherein the EBV-specific CTLs are obtained from a second subject, wherein the second subject is infected with EBV but does not have NPC or HD.

31. A method as claimed in claim 26 wherein the EBV-specific CTLs are LMP1 and/or LMP2-specific CTLs.

32. A method of reducing the risk of infectious mononucleosis or post transplantation lymphoproliferative disease in a subject which method includes administering to the subject an effective amount of:-

(1) at least one CTL epitope as claimed in any one of claims 1 to 3;

(2) a subunit vaccine as claimed in any one of claims 4 to 10;

(3) a nucleic acid sequence as claimed in any one of claims 11 to 13;

(4) a vector as claimed in any one of claims 14 to 16;

or

(5) a polypeptide as claimed in claim 17.

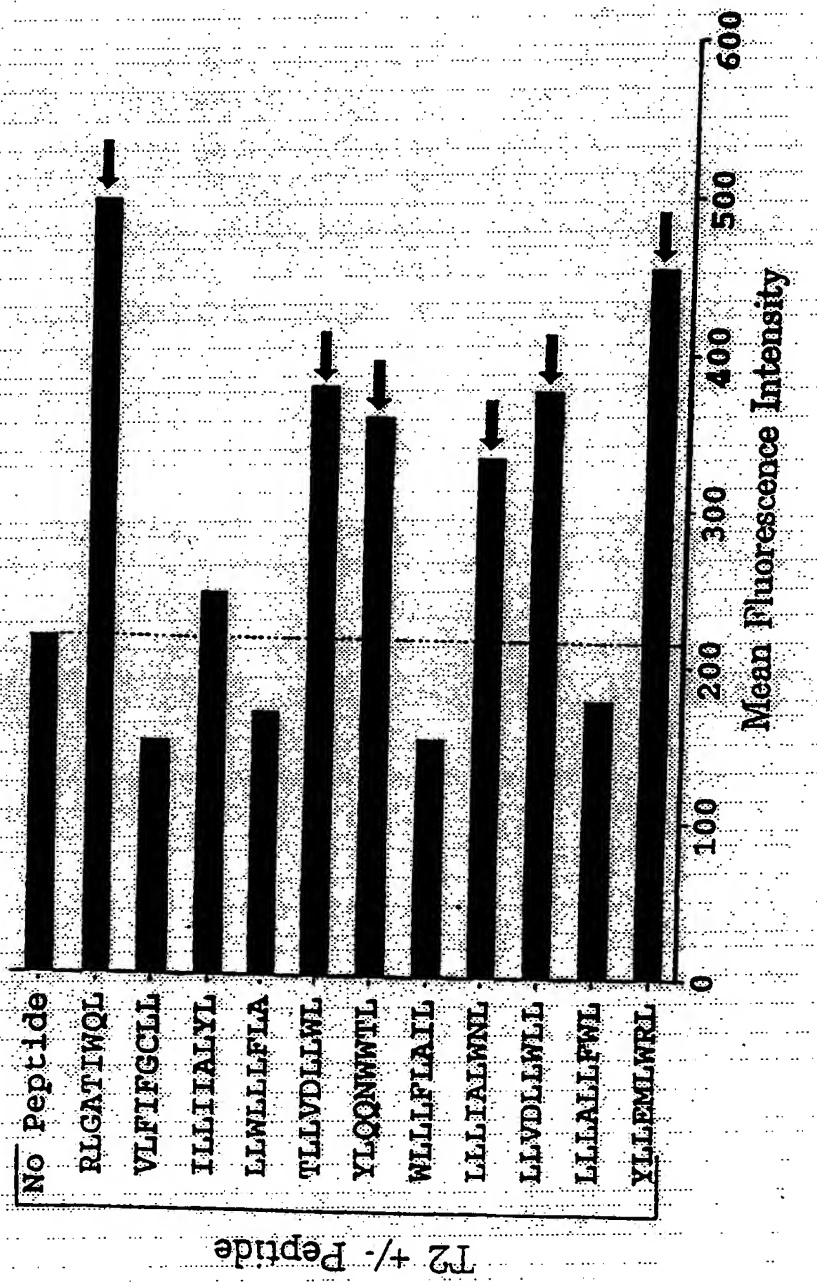
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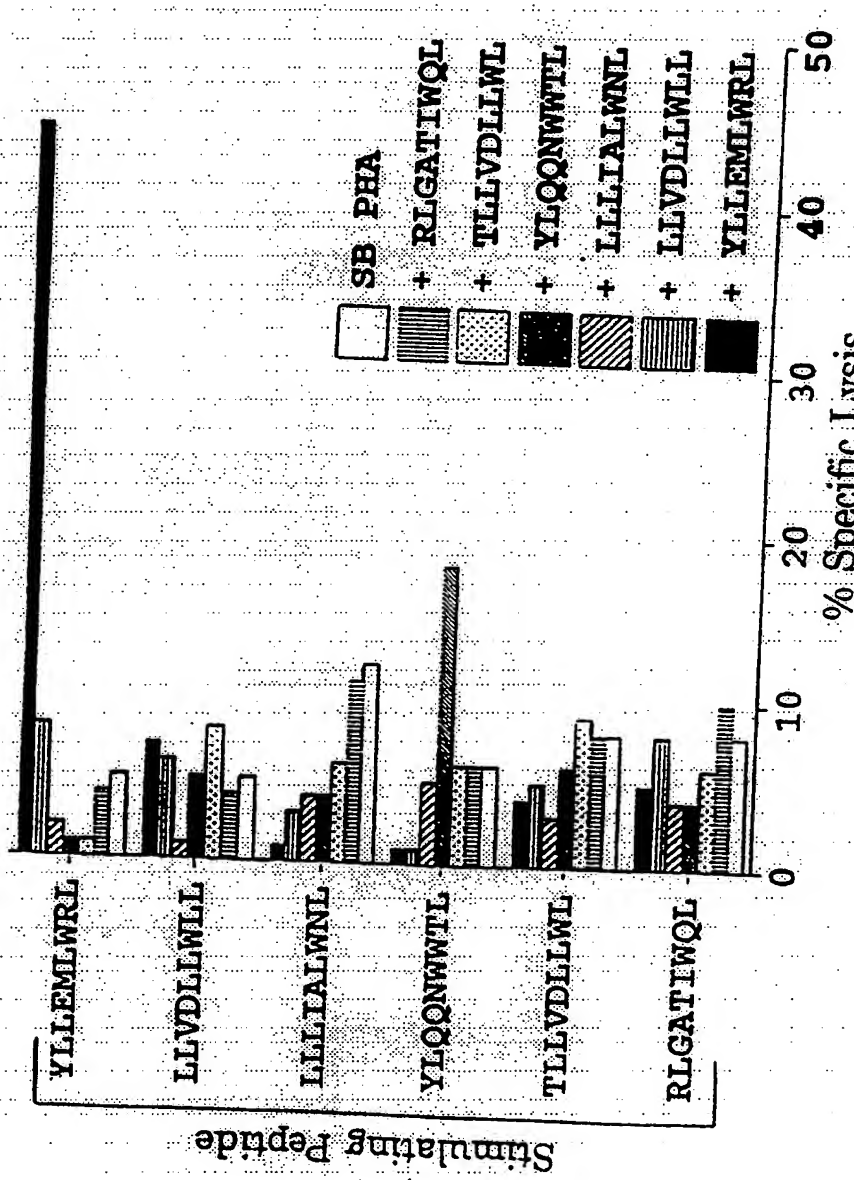
Figure 1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+ TLLVDLLWL
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+ YLLEMLWRL
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10
20
30
40
50
% Specific Lysis
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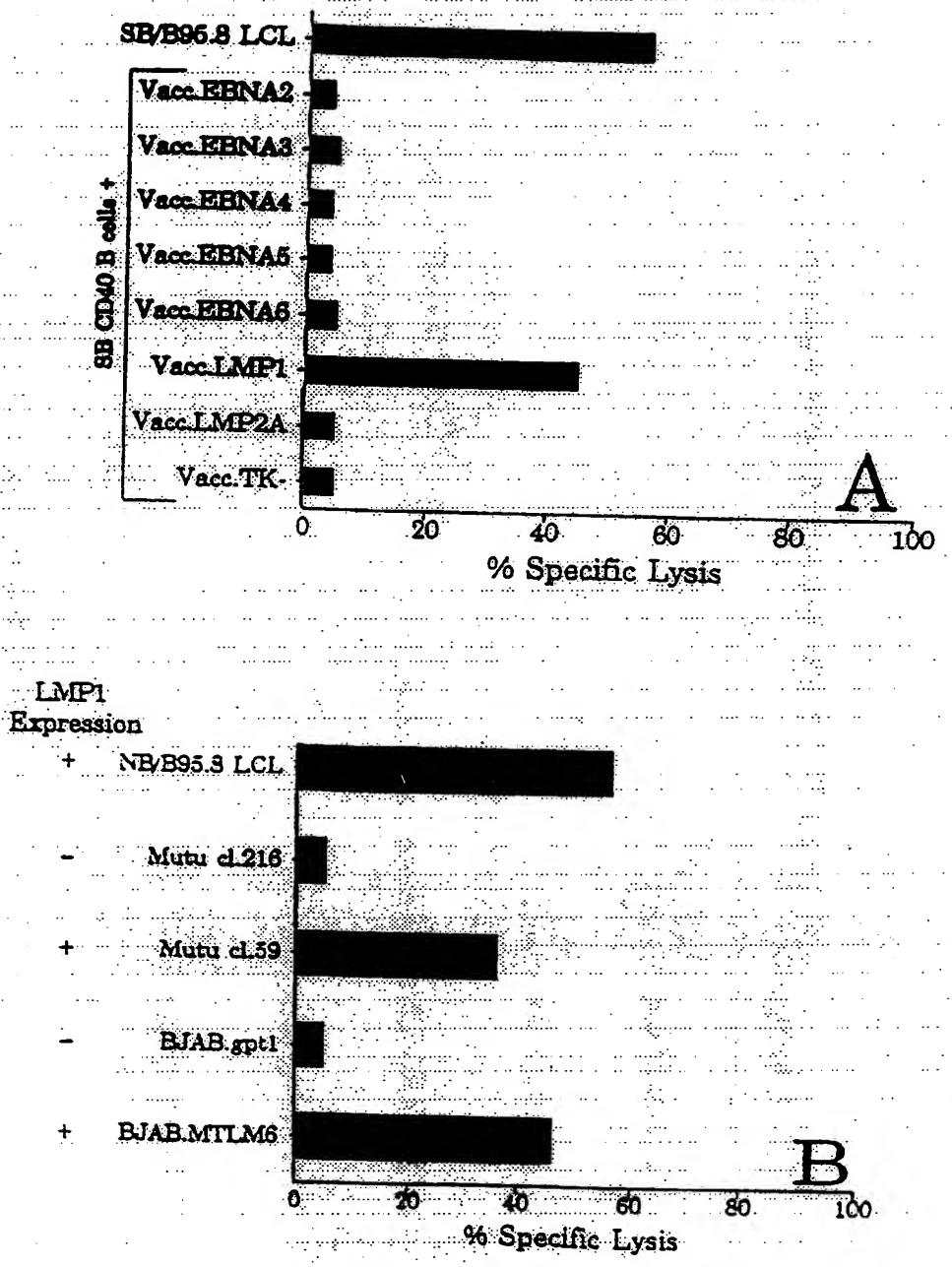
Stimulating Peptide

Figure 2


SB/B96.8 LCL
Vacc.EBNA2
Vacc.EBNA3
Vacc.EBNA4
Vacc.EBNA5
Vacc.EBNA6
Vacc.LMP1
Vacc.LMP2A
Vacc.TK-
SB CD40 B cells +
0
20
40
60
80
100
% Specific Lysis
A
LMP1 Expression
+ NB/B95.8 LCL
- Mutu cl.216
+ Mutu cl.59
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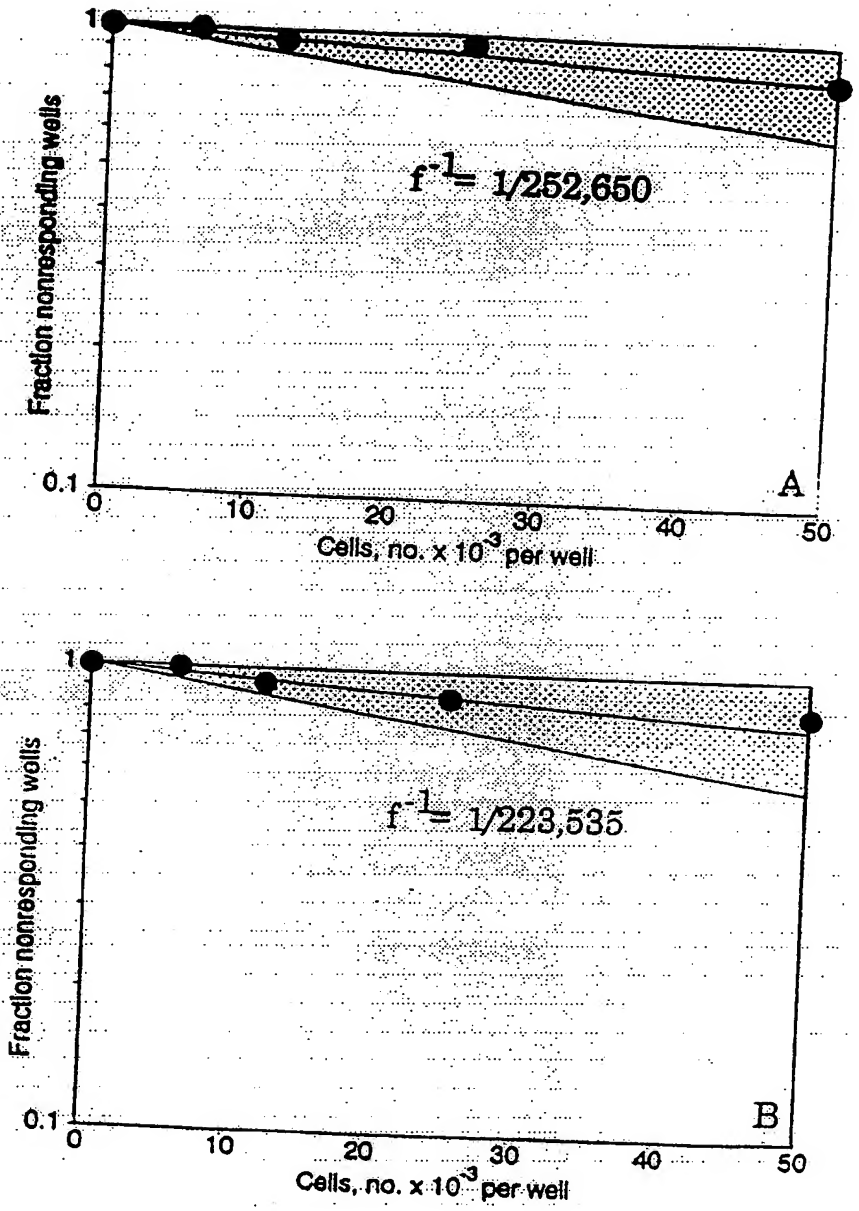
20
40
60
80
100
% Specific Lysis
B

Figure 3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Fraction nonresponding wells
f^{-1}= 1/252,650
A
0
10
20
30
40
50
Cells, no. x 10^{-3} per well
1
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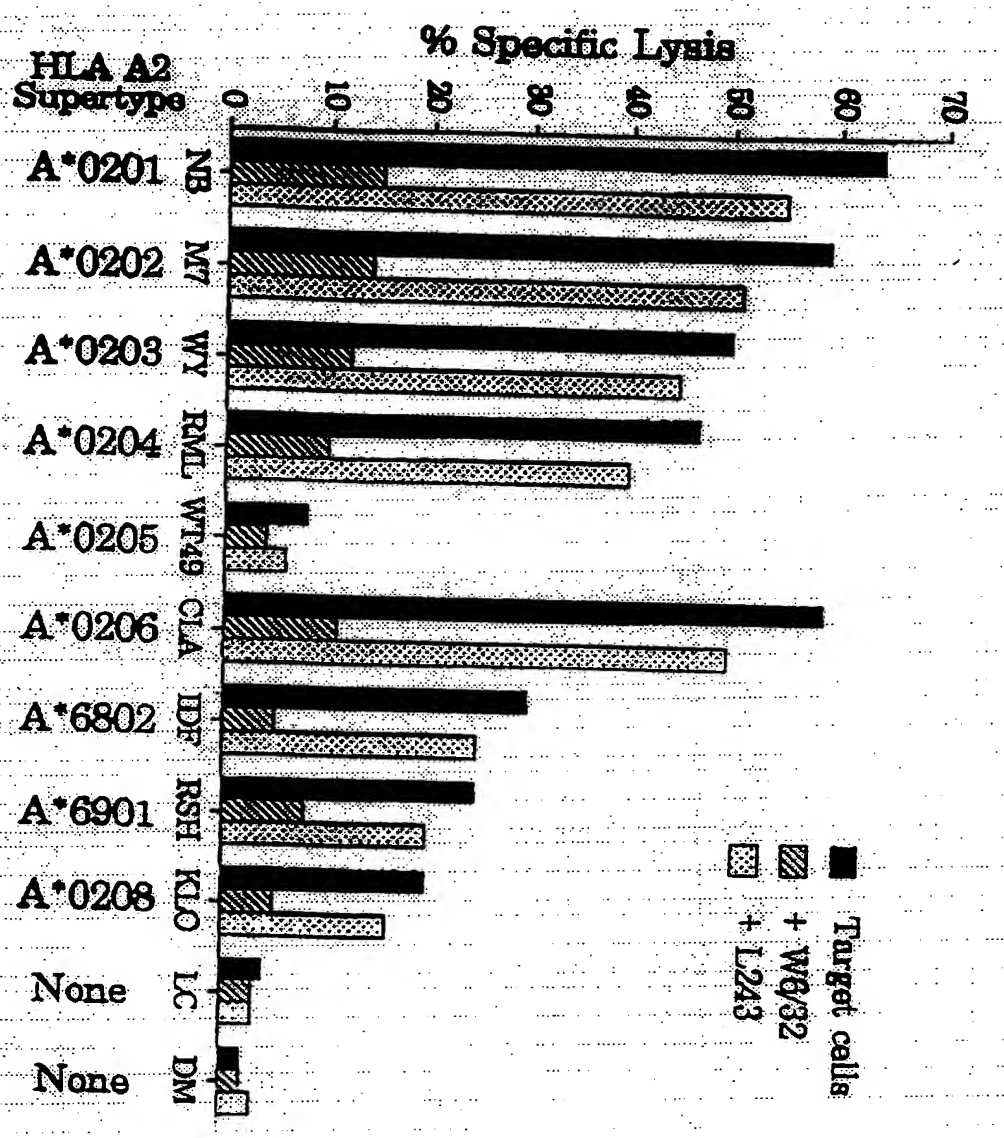
30
40
50
Cells, no. x 10^{-3} per well

Figure 4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20
30
40
50
60
70
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A*0202
A*0203
A*0204
A*0205
A*0206
A*6802
A*6901
A*0208
None
None
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MT
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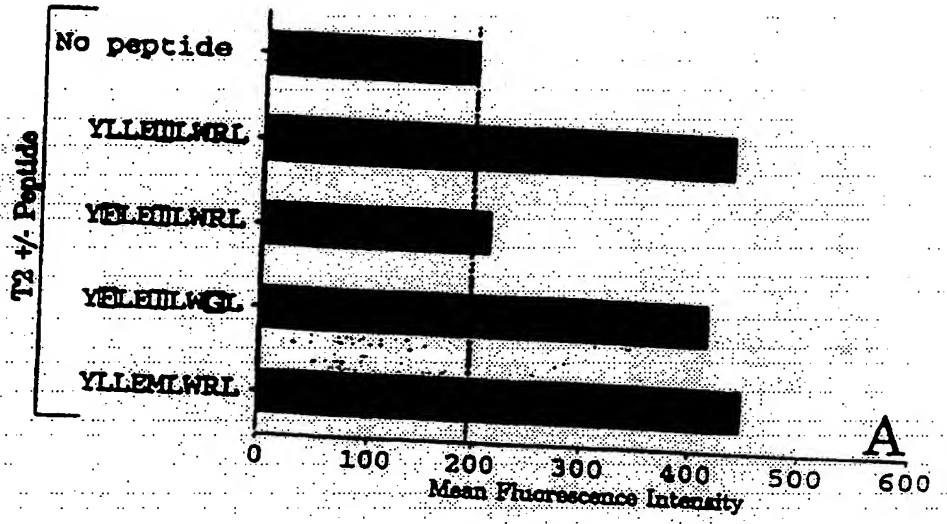
WT49
CLA
IDF
RSH
KLO
LC
DM
Target cells
+ W6/32
+ L243

Figure 5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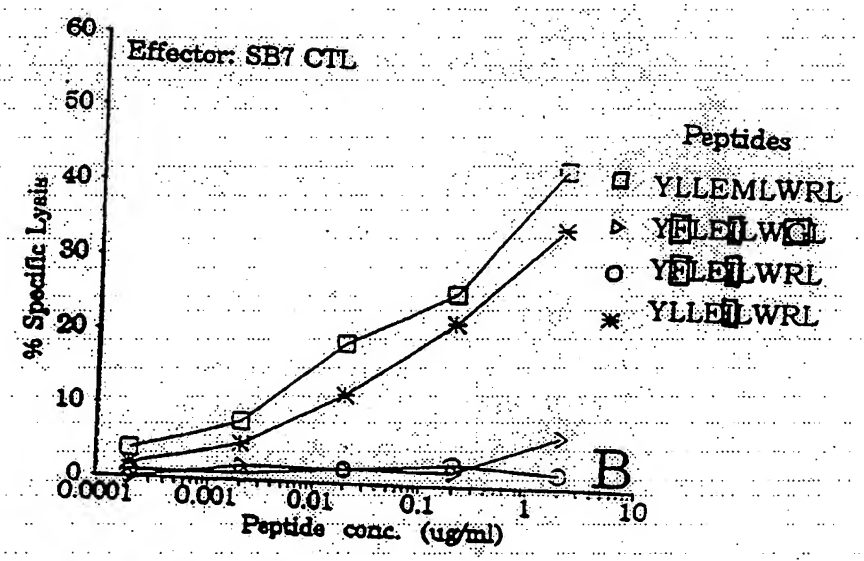
Mean Fluorescence Intensity
A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50
40
30
20
10
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0.001
0.01
0.1
1
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Peptide conc. (ug/ml)
Peptides
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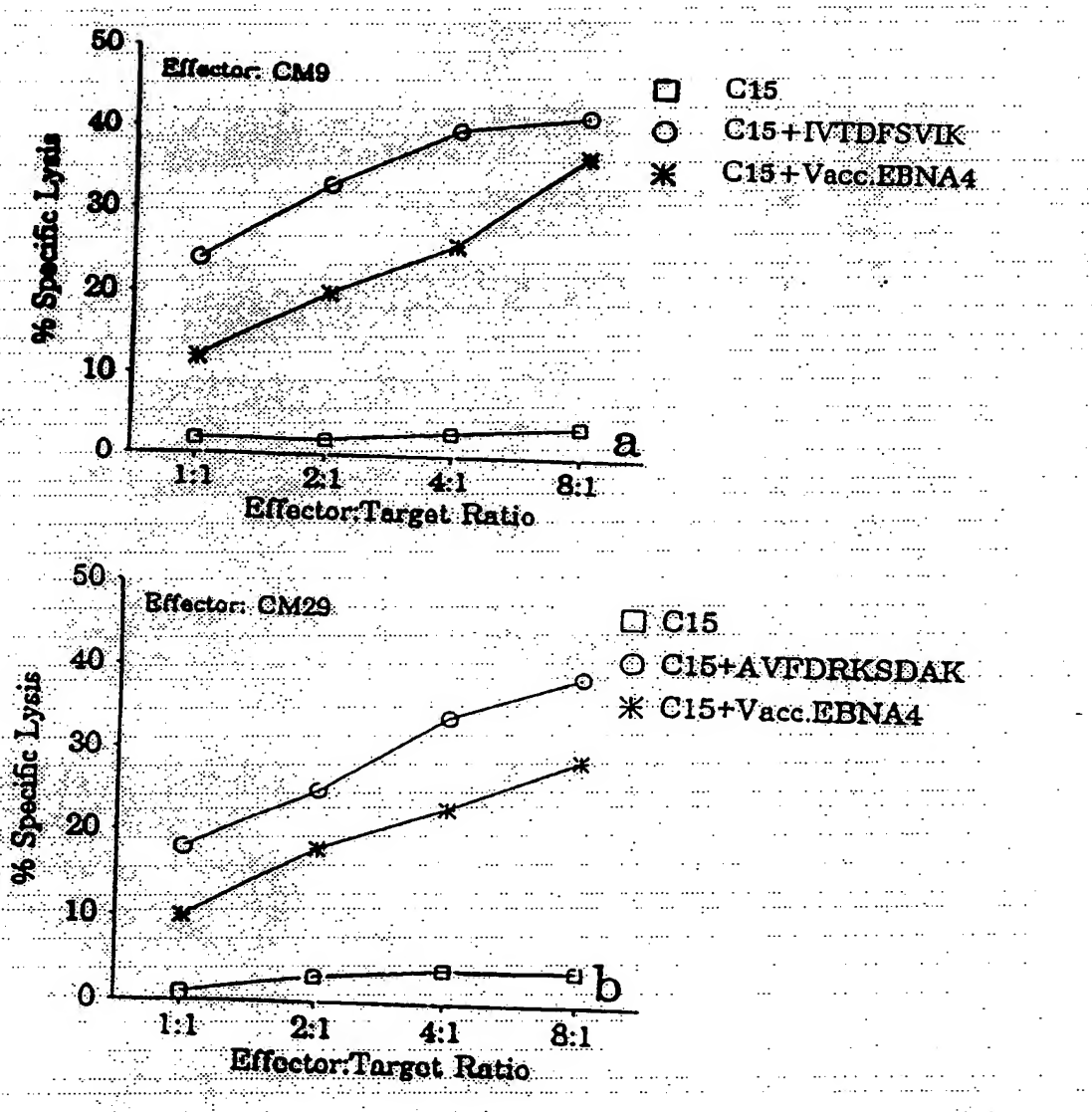
YELEMLWRL
YLLEMLWRL
B

Figure 6


Effector: CM9
% Specific Lysis
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40
30
20
10
0
1:1
2:1
4:1
8:1
Effector:Target Ratio
C15
C15+IVTDFSVIK
C15+Vacc.EBNA4
a
Effector: CM29
% Specific Lysis
Effector:Target Ratio
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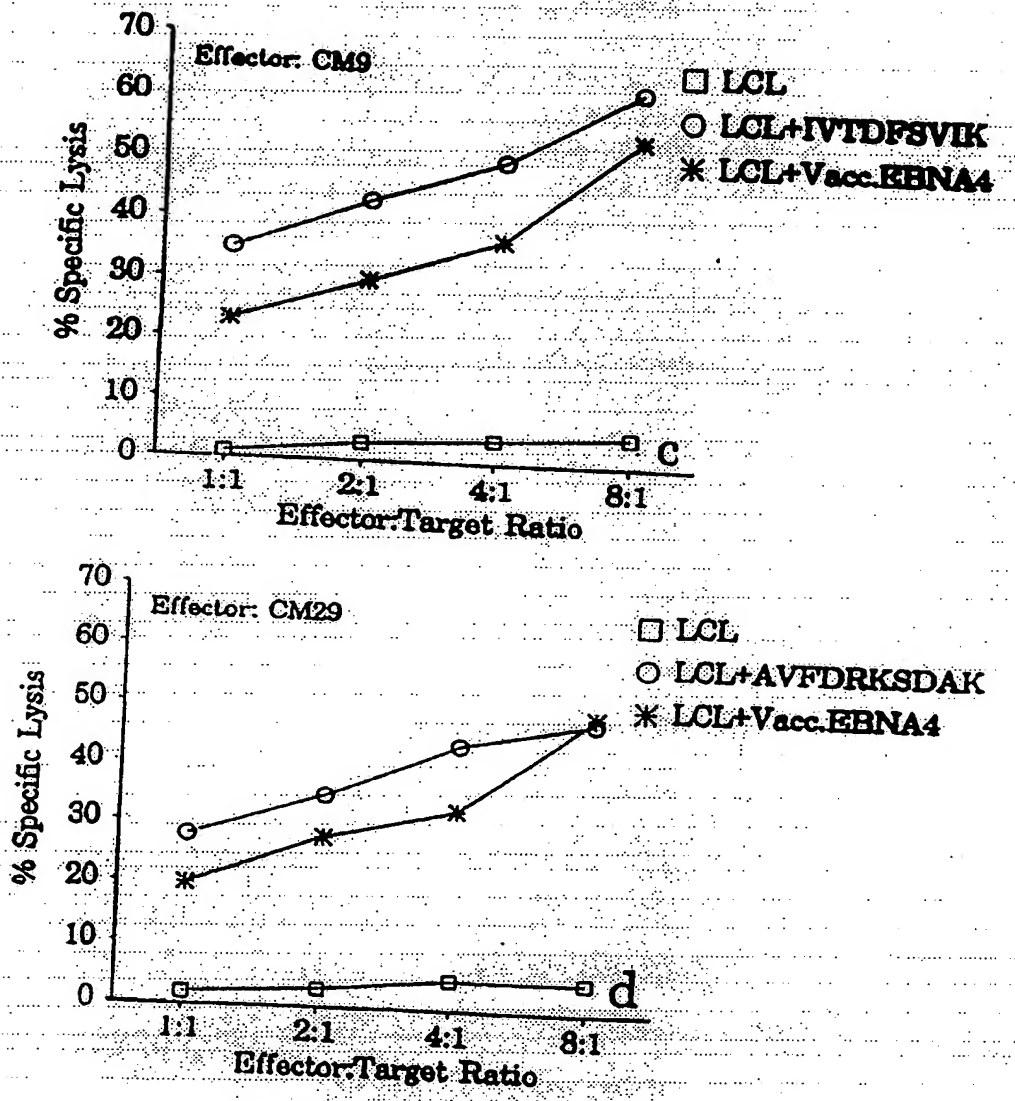
C15+AVFDRKSDAK
C15+Vacc.EBNA4
b

Figure 7



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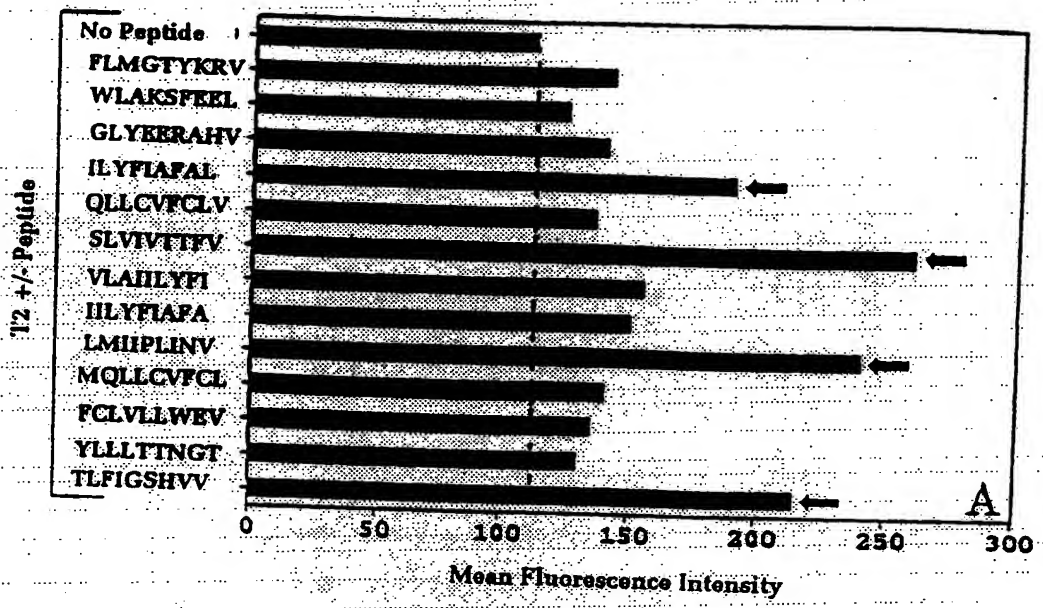
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LMIIPLINV
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100
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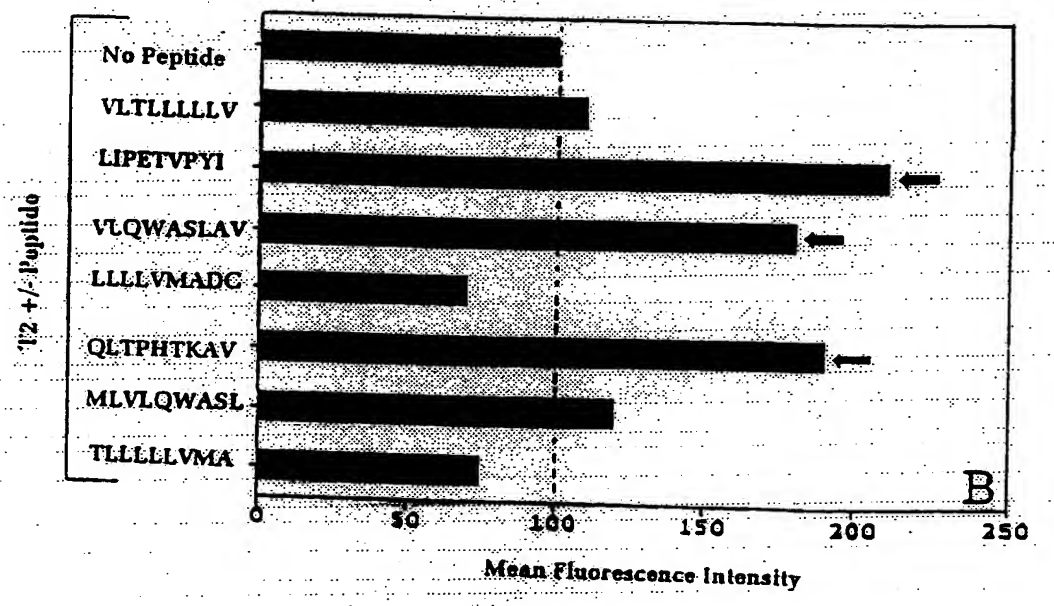
200
250
300
Mean Fluorescence Intensity
A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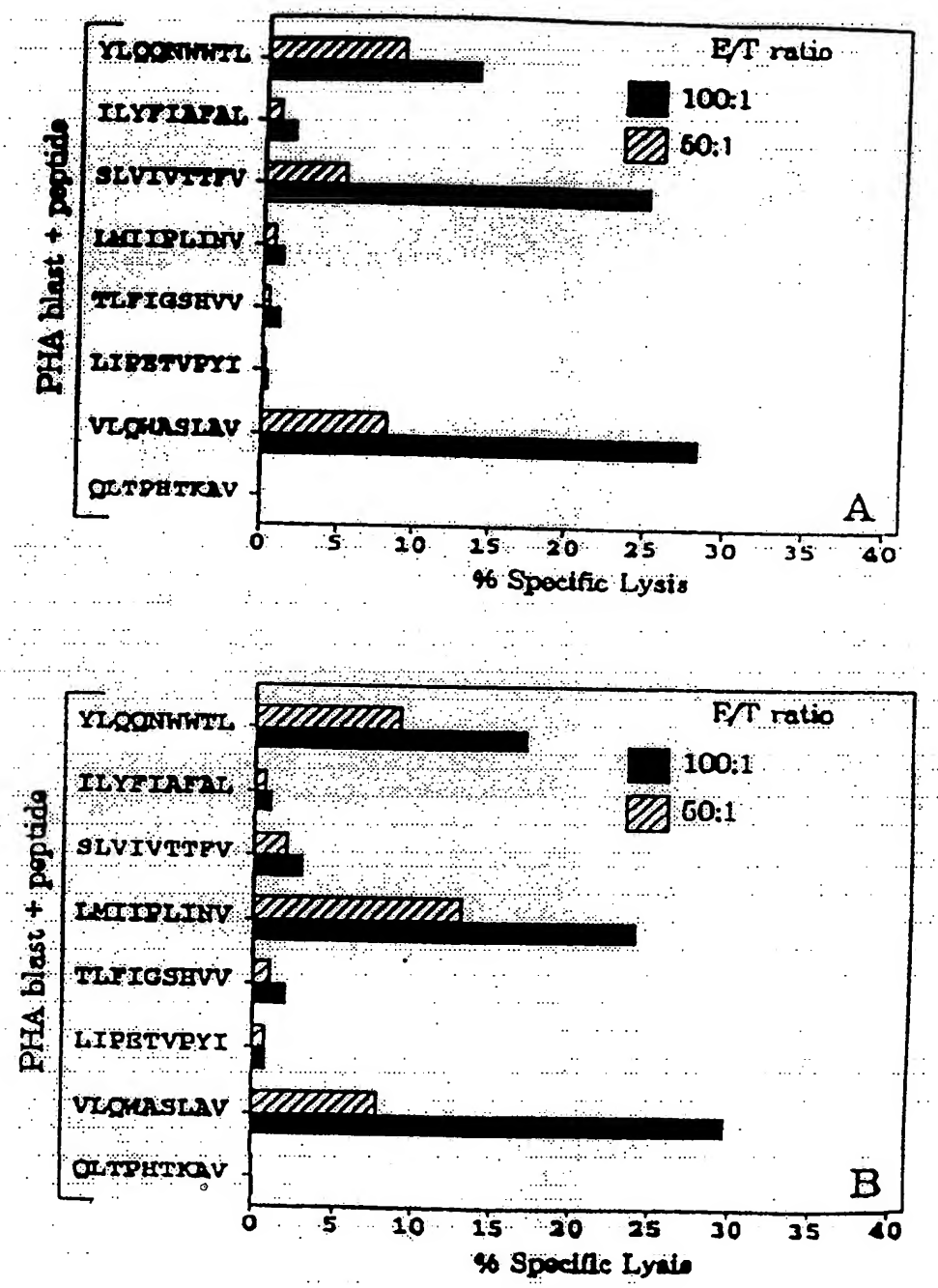
0
50
100
150
200
250
Mean Fluorescence Intensity
B

Figure 8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50:1
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YLQQNWWTL
ILYFIAFAL
SLVIVTTFV
LMIIPLINV
TLFIGSHVV
LIPETVPYI
VLQHASLAV
QLTPHTKAV
A
0 5 10 15 20 25 30 35 40
% Specific Lysis
E/T ratio
100:1
50:1
PHA blast + peptide
YLQQNWWTL
ILYFIAFAL
SLVIVTTFV
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B
0 5 10 15 20 25 30 35 40
% Specific Lysis

Figure 9

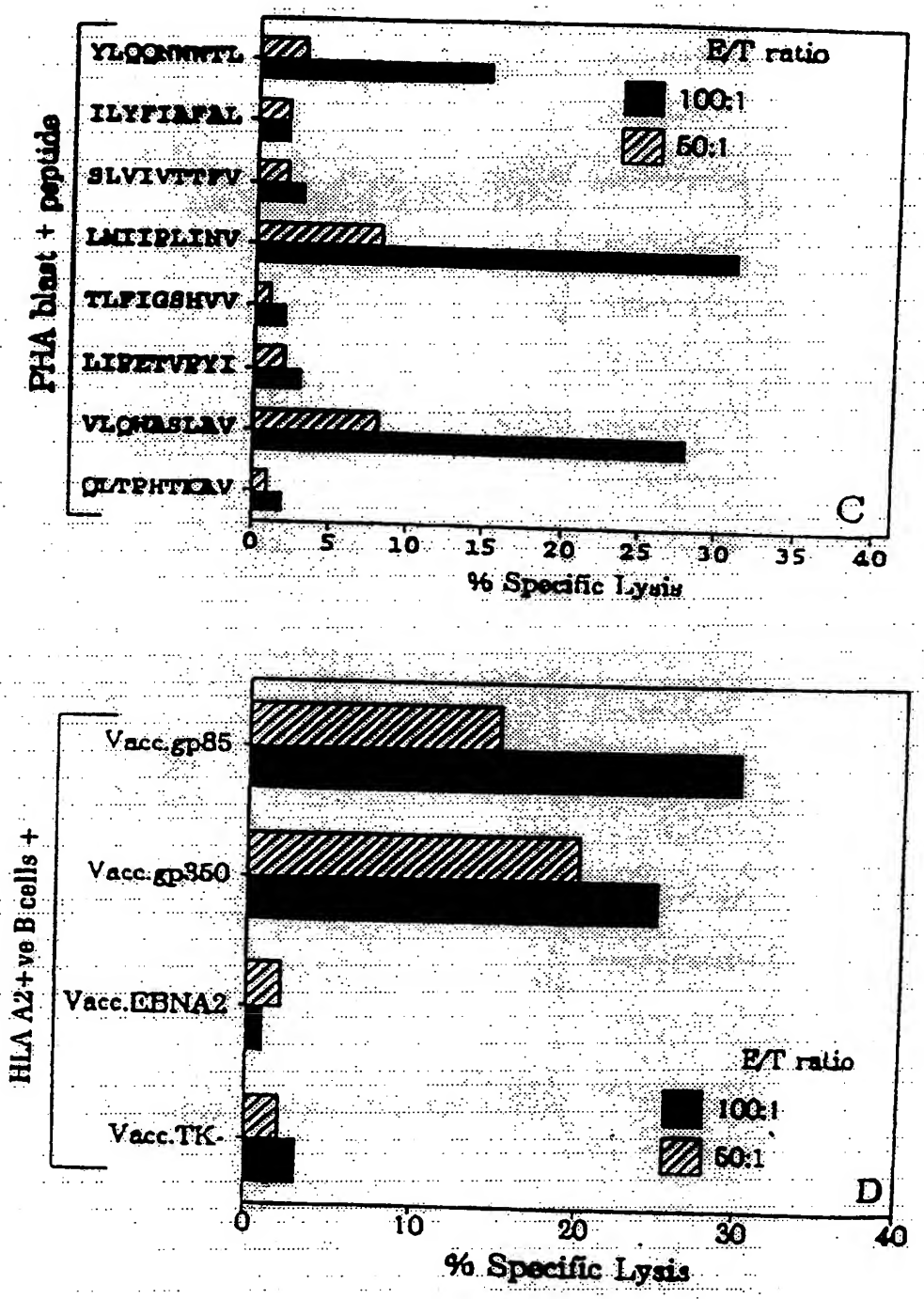
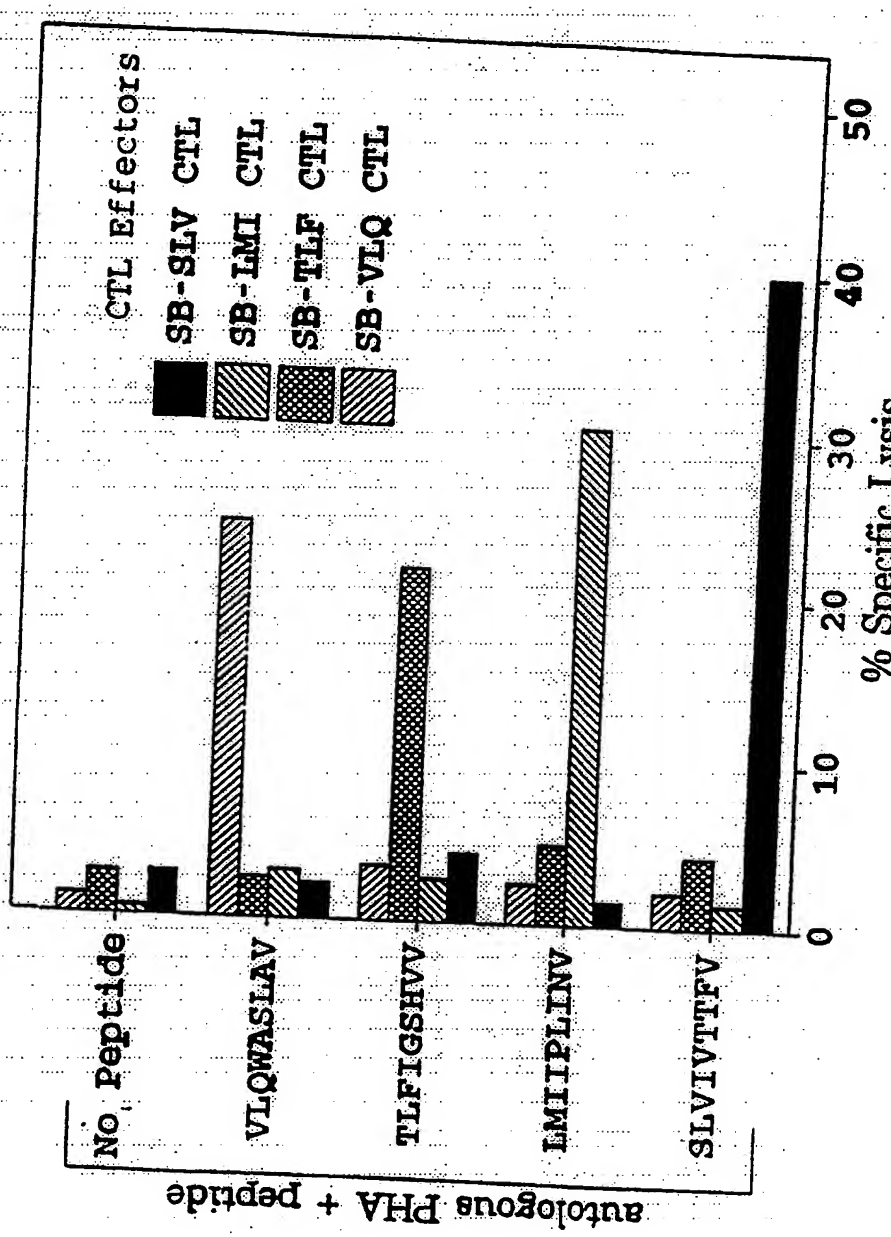


Figure 9 (continued)


CTL Effectors
SB-SLV CTL
SB-LMI CTL
SB-TLF CTL
SB-VLQ CTL
% Specific Lysis
0
10
20
30
40
50
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LMIIPLINV
SLVIVTFV
autologous PHA + peptide

Figure 10

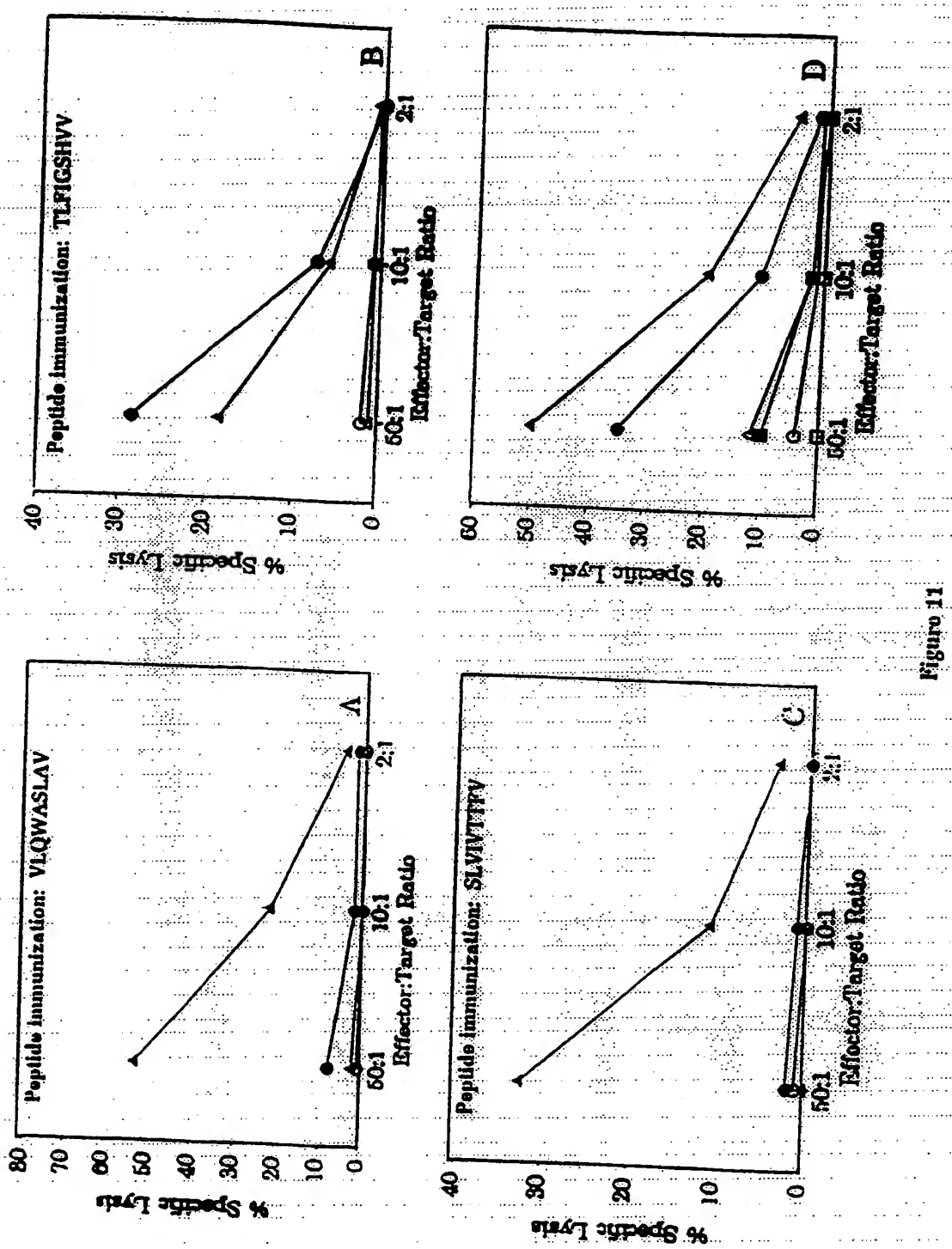
B
Peptide immunization: TLFIGSHVV
% Specific Lysis
Effector:Target Ratio
2:1
10:1
50:1
D
A
Peptide immunization: VLQWASLAV
C
Peptide immunization: SLVIVTTFV

Figure 11

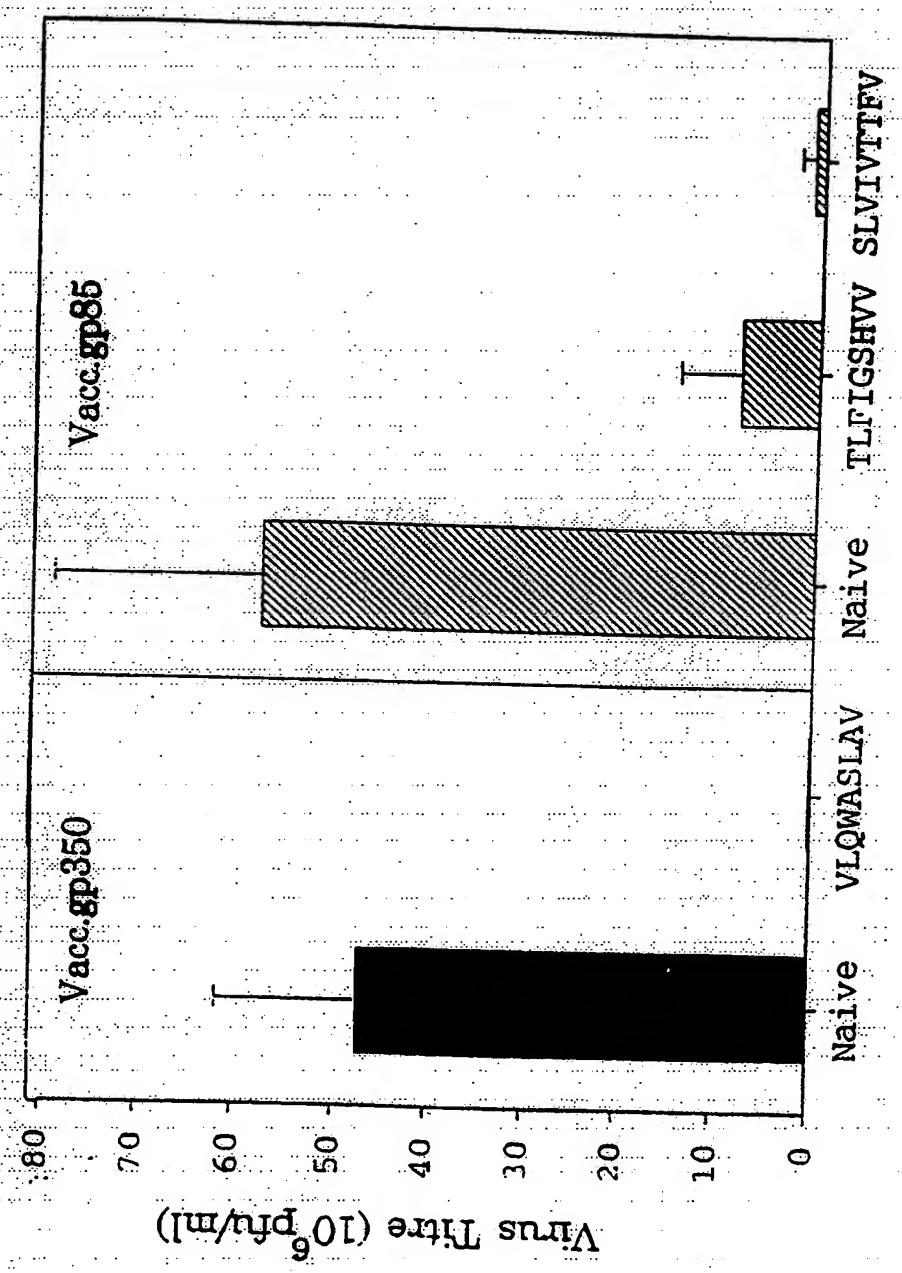
Virus Titre (10^6 pfu/ml)
0
10
20
30
40
50
60
70
80
Vacc.gp350
Naive
VLQWASLAV
Vacc.gp85
Naive
TLFIGSHVV
SLVIVTTFV

Figure 12

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00531

A. CLASSIFICATION OF SUBJECT MATTER

Int Cl[6]: C07K 7/06; C07H 21/04; A61K 39/245, A61K 39/295

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC[6]: C07K 7/06; C07H 21/04; A61K 39/245, A61K 39/295

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE - "cytotoxic Epstein-Barr virus T-cell epitope"
CHEMICAL ABSTRACTS - PEPTIDE SUBSTRUCTURE SEARCH

C. DOCUMENTS COSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,Y A	THORLEY-LAWSON, D A et al PROC NATL ACAD SCI USA volume 84 pages 5384-5388 see in particular Figure 1	1,2,17 3-16,18-32
X,Y A	OBA, D E et al JOURNAL OF VIROLOGY, April 1988 volume 62 No. 4 pages 1108-1114 see in particular Figure 1	1,2,17 3-16,18-32
X,Y A	BURROWS, S R et al JOURNAL OF EXPERIMENTAL MEDICINE January 1990 volume 171 pages 345-349 see whole document	1,2,17 3-16,18-32

[X] Further documents are listed in the continuation of Box C

[X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 September 1998	15 SEP 1998
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200 WODEN ACT 2606 AUSTRALIA Facsimile No.: (02) 6285 3929	Authorized officer K.G. ENGLAND Telephone No.: (02) 6283 2292

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INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00531

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X,Y A	BURROWS, S R et al JOURNAL OF VIROLOGY August 1990 volume 64 No: 8 pages 3974-3976 see especially page 3974	1,2,17 3-16,18-32
X,Y A	LI-FU HU et al JOURNAL OF GENERAL VIROLOGY 1992 volume 72 pages 2399-2409 see especially Figure 6	17 1-16,18-32
X,Y A	APOLLONI, A et al EUROPEAN JOURNAL OF IMMUNOLOGY 1992 volume 22 pages 183-189 see especially page 183, page 188 end column 2, Tables 3 and 4	1,4,17 2,3,5-16,18-32
X,Y A	LEES, J F et al VIROLOGY 1993 volume 195 pages 578-586 see especially Figure 1, end column 1 page 584, "Discussion"	17,19 1-16,18,20-32
X,Y A	POTHEN, S et al INT JOURNAL OF CANCER 1993 volume 53 pages 199-204 see especially Table 1	1,2 3-32
X,Y A	SUHRBIER, A et al PEPTIDE RESEARCH 1995 volume 8 No: 5 pages 258-252 see especially the Abstract, results	1,2,17 3-16,18-32
X,Y A	BURROWS, J M et al JOURNAL OF VIROLOGY April 1996 volume 70 No: 4 pages 2490-2496 see especially Table 1	1,2 3-30
X A	MACKETT, M et al JOURNAL OF MEDICAL VIROLOGY 1996 volume 50 pages 263-271 see page 264 column 1, Table II in particular	17,19 1-16,18,20-30
X,Y A	FRANKEN, M et al JOURNAL OF VIROLOGY, November 1996 volume 70 no: 11 pages 7819-7826 see Abstract, Figure 1 in particular	17 1-16,18-32
X,Y A	KERR, B M et al JOURNAL OF VIROLOGY December 1996 volume 70 No: 12 see especially Abstract, Figures, page 8863 column 1	1,2,4,17 3,5-16,18-32
Y	FIELDS, B N (Ed) FUNDAMENTAL VIROLOGY, 3rd edition page 1121 column 2 RAVEN PRESS, New York 1995	1-32

INTERNATIONAL SEARCH REPORT

International Application No.
PCT/AU 98/00531

C (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X A	EP 173254 A1 (WOLF, HANS-JOACHIM) 5 March 1986 see Figure 17	17 1-16,18-32
X Y	AU 19425/95 A1 (THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH et al) 21 September 1995 see pages 1, 2, 3, 11, 12 and claims	1,4,8,9,10,11,17,18,19-32 1-32
X Y	AU 30723/95 (THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH et al) 8 February 1996 see Example 1, claims, pages 1-5	1,11,14,16,17,18,19 1-32
P,X P,Y	AU 27590/97 (THE COUNCIL OF THE QUEENSLAND INSTITUTE OF MEDICAL RESEARCH et al) 4 December 1997 see Tables 3, 4, pages 1-5	1,4,7,8-11,14-17,19,32 1-32
A	AU 82073/87 A (SCRIPPS CLINIC AND RESEARCH FOUNDATION) 9 June 1988	

INTERNATIONAL SEARCH REPORT
Information on patent family members

International Application No.
PCT/AU 98/00531

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
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		JP	61257188	JP	8191695	CN	86100441
AU	19425/95	WO	95/24925	GB	2301591	JP	10501682
		CA	2185540	CA	2185541	WO	95/24926
AU	30723/95	WO	96/03144	EP	769963	JP	10506004
		CA	2195642				

END OF ANNEX